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Introduction

The overall objective is to test the role of BRMS1 in the ability of breast xenografts to metastasize to bone following intracardiac injection. A corollary is that BRMS1-regulated genes, especially osteopontin (OPN) will influence metastasis. Briefly, OPN is thought to be a metastasis promoting gene, while BRMS1 is a metastasis suppressor. Restoration of BRMS1 expression suppressed OPN.

Knowing that BRMS1 suppresses metastasis from an orthotopic site to lung and regional lymph nodes (Seraj et al., 2000; Samant et al., 2001; Shevde et al., 2002), it is not known whether metastasis is suppressed all sites. Since breast cancer spreads most commonly to bone, we will test whether BRMS1 and/or molecules regulated by BRMS1 block bone metastasis.

Summary of Progress

In order to accomplish the planned experiments, better bone metastasis models for breast cancer were needed. At the time of the original submission, we had tagged some melanoma cells with green fluorescent protein and showed increased ability to detect lesions at multiple sites, including bone. Subsequently, we tagged MDA-MB-435 human breast carcinoma cells with GFP and recently published the utilization of those cells for assessing bone metastasis (Harms and Welch, 2003).

Our GFP-tagged cells have been distributed widely and many labs have begun using them for their studies. However, in the meantime, we encountered gene silencing in the vectors used (CMV promoter driven GFP), making long-term studies more challenging to interpret. Therefore, we have begun infections using other vectors and other promoters. New variants appear more stable and the metastatic potentials are being verified.

BRMS1 is part of a histone deacetylase complex (Meehan et al., 2004); so, it follows that it might be regulating effector molecules. In a multiple microarrays, a prominent change was down-regulation of osteopontin (OPN), a molecule known to promote metastasis (Oates et al., 1997; Debies and Welch, 2001; Yeatman and Chambers, 2003). OPN reduction was validated using real-time PCR (RTQ) and immunoblotting. A question to be addressed in this grant is whether OPN is a downstream regulator of metastasis. Originally, we proposed to over-express OPN but opted to try small interfering RNAs (RNAi) (McManus and Sharp, 2002; Ramaswamy and Slack, 2002) to decrease OPN expression as a priority. Initial experiments showed that diminished OPN expression suppressed not only metastasis, but tumorigenicity as well. Additional controls need to be done in order to make definitive conclusions. In the meantime, we decided to work with Dr. Ann Chambers, internationally recognized for her work on OPN, in a collaboration to re-express OPN in BRMS1-transfected breast carcinoma cells.

We have also assessed the osteopontin (OPN) 'receptor,' $\alpha v\beta 3$ integrin, in the development and/or progression of bone metastasis (Harms et al., 2004). $\alpha v\beta 3$ integrin has been implicated in tumor cell adhesion as well as osteoclast adhesion events. Therefore, we evaluated whether disruption of the interaction would lead to suppression of metastasis by one or both steps. $\alpha v\beta 3$ integrin appears to be more relevant in early stages of bone metastasis in our model.

Original Statement of Work

Task 1: Develop stable fluorescent cell lines
- Transfect 435BRMS1 and 231BRMS cells with GFP and dsRED
- Transfect neo11/435 cells with GFP and dsRED
- Select highly fluorescent subpopulations by fluorescence activated cell sorting

Task 2: Restore OPN expression in poorly metastatic cells
- Transfect 435BRMS1^{GFP}, 231BRMS1^{GFP} and neo11/435^{GFP} with OPN
- Select low, medium and high expressing clones

Task 3: Test metastatic potential of transfectants (injections and histological examination)
Task 4: Test tumor cell - osteoblast (hFOB) interactions
Task 5: Test tumor cells - sinusoidal endothelium (HBME) interactions

Key Research Accomplishments (*New since the previous progress report*)

Task 1: Develop stable fluorescent cell lines

GFP-tagged MDA-MB-435 cells were generated and metastatic properties validated in the first report. The patterns were identical to those of the parent. The lines were generally quite stable; however, a portion (occasionally up to 30%) of the population was silenced for GFP expression, making interpretation more difficult (i.e., metastasis would have been under-reported). In the previous progress report, we proposed to utilize a Tet-inducible system developed by Dr. T.C. He. For reasons that we cannot explain, the system works fine for transient transfections, but we (and other colleagues) have never obtained stable transfectants). Therefore, we are using new vectors and promoters to overcome this problem. Colleagues have helped us infect with *Lentivirus* vectors and the expression/stabilization issues seem to have been resolved. Expressing cells were sorted for the top 25% intensity by FACS. The variants appear to be quite stable. Mice injected with the new GFP-tagged variants are being evaluated at this time. At approximately one-third through the experiment, tumor growth rates are comparable.

We planned to begin work with dsRed; however, this has been delayed while GFP parameters are optimized. Vectors containing other suppressors and fluorescent tags are being designed and prepared.

Task 2: Restore OPN expression in poorly metastatic cells

Re-expression experiments were proposed and are reasonable; however, double or triple transfections were more difficult than anticipated. While we got expression, levels were substantially below those in the parental cell line. We discussed this with Dr. Ann Chambers, who has experienced similar difficulties. Therefore, we opted for our contingency strategy. We have designed RNAi to decrease OPN expression in parental cells. In general, we believe that this approach is better because it more closely recapitulates what is occurring when BRMS1 is re-expressed (i.e., OPN levels will decrease).

RNAi studies were initiated using the pSUPER stable transfection vector. We were able to achieve >90% reduction in OPN expression in MDA-MB-435 cells and the effect was not due to induction of interferon response. Selectivity was evident in that eIF2α and β-actin levels were not affected. The OPN-suppressed cells were evaluated for motility and invasion in vitro, growth, growth in soft agar, tumorigenicity and metastasis. While migration and invasion generally correlated inversely with OPN expression,

Table 1: Constitutive expression of siRNA to osteopontin in MDA-MB-435 cells suppresses tumorigenicity and metastasis.

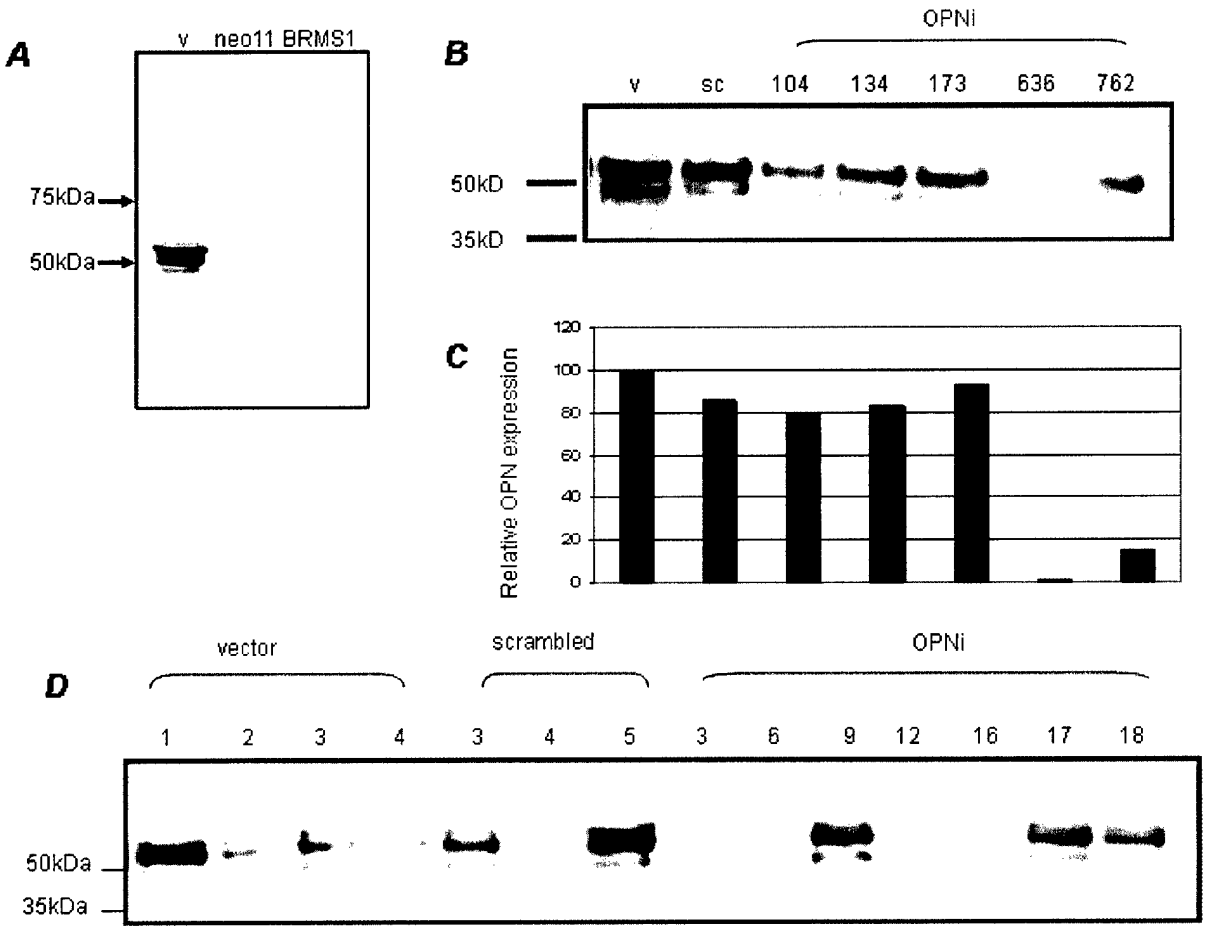
	Incidence	
Cell Line	Tumor	Lung metastases
MDA-MB-435	100	90
Vector-1	100	90
Vector-2	100	80
Vector-3	100	90
Vector-4	100	80
Scrambled-3	90	80
Scrambled-5	90	80
OPN-3	20	25
OPN-6	20	30
OPN-12	100	90
OPN-16	100	80

adhesion did not. Serum OPN was measured in mice bearing orthotopic tumors; however, two clones showing the greatest suppression of OPN were also tumor suppressed! Intuitively, this made no sense with regard to OPN being downstream of BRMS1. Therefore, we have to revise our hypothesis. In addition, we are working to add new 'controls' for the recently found non-specificities associated with RNAi/siRNA (Reynolds et al., 2004; Samuel, 2004).

Task 3: Test metastatic potential of transfectants (injections and histological examination)

In order to fully understand the effects of OPN and BRMS1 on metastasis, it has become increasingly apparent that baseline information about bone metastasis development is largely unknown. For example, what are the kinetics of tumor cell arrival, transit to the trabecular space, initiation of proliferation, osteoclast recruitment, osteoblast inhibition, etc.? If OPN and BRMS1 affect bone metastasis, it would be important to understand at which step(s). Therefore, we began a study to understand the early events in bone colonization.

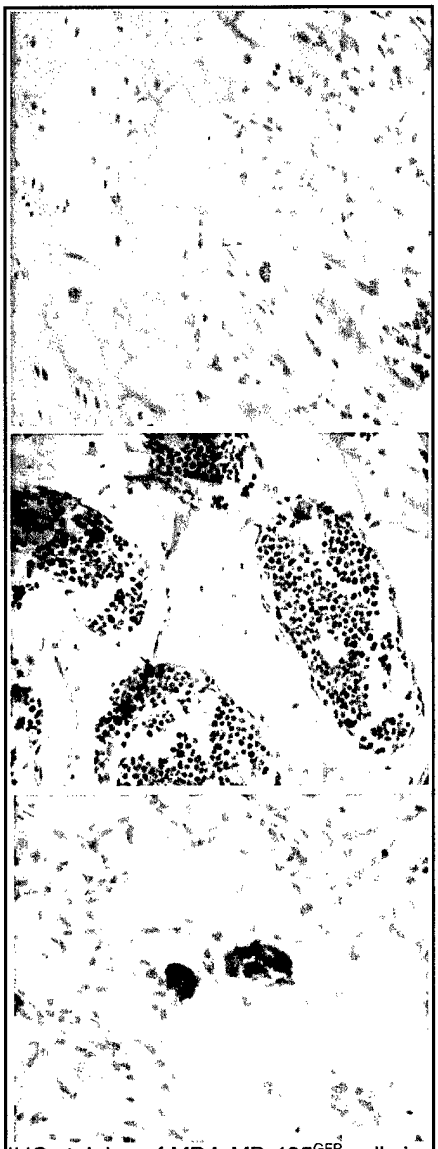
MDA-MB-435^{GFP} cells were injected into the left ventricle of athymic mice. At 1, 2, 4, 8, 24, 48, 72 hr and 1, 2, 4, 6 wk post-injection, mice were euthanized (n=5-10, depending upon time point) and femurs



(A) Immunoblot validating microarray data showing that OPN expression is reduced in neo11/435 and BRMS1-transfected MDA-MB-435 cells compared to vector-only (v) cells. **(B)** Western blot comparing OPN in cell-free conditioned medium from transiently transfected MDA-MB-435 cells to compare various heteroduplexes for efficiency of suppression. Transfections were done with V: pSUPER only, SC: universal scrambled heteroduplex, and heteroduplexes beginning at nucleotides 104, 134, 173, 636 or 762. **(C)** Densitometry of panel B. **(D)** Stably expressing clones were generated using the pSUPER-NM636 heteroduplex and an immunoblot of cell-free conditioned medium using anti-OPN shows significant suppression of OPN expression.

collected, fixed in paraformaldehyde (4%, 1-5 day) and decalcified (500 mM EDTA, 1-5 day). Random bones were flushed to isolate cells for flow cytometry. Paraffin-embedded tissues were sectioned and stained (H&E). Interim results are as follows.

Clusters of cancer cells were readily identified ≥2 wk post-injection using H&E staining. However immunohistochemistry (anti-GFP rabbit Ig; 1:250; Molecular Probes) was used to identify single cells at earlier time points. Single tumor cells are observed in bone sections as early as 1 hour post- injection. Virtually all cells were detected in the metaphyseal end of the bone. Few cells, either single or in clumps were seen in bone marrow. In one bone, a group of cells was located beneath the periosteum. Our initial interpretation is that tumor cells disseminate widely in the bone, but preferentially localize to the metaphysis. We speculate that femoral blood supply may be explain the location (i.e., the nutrient artery which is the main blood supply of the femur carries most of the blood to the ends of the bone along with branches of the metaphyseal artery). Our working model suggests that cancer cells do not begin proliferating until >72 hr (i.e., they remain as single cells prior to 48-72 hr). By one week, tumor cells clumps (2-10 cells) are seen which get progressively larger and form macroscopic metastases by 4-6 weeks. Ongoing studies involve TRAP staining (to determine osteoclasts associated with the tumor mass) and TUNEL staining (to assess tumor cell-induced osteoblast apoptosis). Preliminary data show that tumor cells are proliferating well prior to clear osteolytic lesions, raising questions regarding growth factor feedback resulting from bone degradation. The figures below show IHC of GFP-tagged 435 cells at 2, 24 and 168 hr post injection.



IHC staining of MDA-MB-435^{GFP} cells in bone at 2, 24 and 168 hr after injection into the left ventricle of the heart. Cells are stained with rabbit anti-GFP antibody.

An alternative approach to testing impact of OPN in bone metastasis. With funding from Pfizer, this DOD contract and the UAB Breast Cancer SPORE, we tested whether a small molecule inhibitor of αvβ3 integrin (S247) would affect metastasis to bone. Briefly, S247 was in clinical trials as an inhibitor of osteoporosis because it inhibits osteoclast ring formation. Mice were treated continuously with an inhibitor (designated S247) at three doses on two different schedules. In short, the results showed that presence of S247 prior to tumor cell colonization of bone would inhibit establishment of metastasis. S247 did not appear to diminish proliferation of tumor cells once they got to bone, however. These results were published recently and a reprint is appended (Harms et al., 2004).

Task 4: Test tumor cell - osteoblast (hFOB) interactions

In order to test tumor cell – osteoblast interactions, it was first necessary to confirm proper function of the osteoblast cell line (hFOB1.19). This cell line has been stably transfected with a temperature sensitive SV40 T-antigen that allows growth at the permissive temperature of 34°C, but at the restrictive temperature of 39°C, cell growth is halted and differentiation induced. Differentiation was validated by alkaline phosphatase expression and mineralization (von Kossa staining).

Upon confirmation of proper osteoblast function, adhesion assays with MDA-MB-435^{GFP} cells to osteoblast monolayers were initiated. Both differentiated and undifferentiated osteoblasts were used. We developed a new image analysis method to count adherent fluorescing cells (~100+ images per

experiment). Customized quantification using commercially available image analysis software (SigmaScan Pro 5.0) automatically counts the fluorescent cells, screening on the basis of size and shape to eliminate any background or debris. The program reduced the time of data analysis from 2-3 days to ~20 minutes. Adhesion was modest to undifferentiated hFOB while adhesion to differentiated hFOB was much more rapid and significant.

Task 5: Test tumor cells - sinusoidal endothelium (HBME) interactions

Unlike MDA-MB-435^{GFP} interactions with osteoblasts, there was only a very slight increase in adherence to HBME was almost non-existent at early time points.

Reportable outcomes:

Publications in peer-reviewed journals

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Presentations

What is the definition of metastasis?. Metastasis Working Group, National Cancer Institute , Bethesda,

MD (2/5)

Metastasis suppressor genes. 5th International Congress of the Genetics, Biochemistry and Physiology of NDP kinase/NM23/AWD, Lexington, KY (10/15)

The BRMS1 metastasis suppressor gene appears to function via interactions with the mSin3:histone deacetylase complex. University of Vermont Cancer Center Symposium. (10/3)

Metastasis as a target of chemoprevention? American Association of Cancer Research, Washington, D.C. (7/11)

Cancer metastasis: What is the next generation of clinical targets?, North Dakota State University COBRE Symposium on Proteinases and Proteinase Inhibitors. Fargo, ND (6/1)

BRMS1: Illuminating a surprising regulatory point for breast cancer metastasis? National Cancer Institute, Metastasis: Prevention or Therapy, Bethesda, MD (5/20)

Metastasis suppressor genes in human cancer, 18th Annual Symposium on the Biological Approaches to Cancer Treatment, Nagoya Japan (5/17)

A novel mechanism of metastasis regulation by the BRMS1 metastasis suppressor gene. University of Tokyo Symposium on Cancer Metastasis – Future Cancer therapy through understanding metastasis (5/16)

Cancer metastasis: What is the next generation of clinical targets?, North Dakota State University COBRE Symposium on Proteinases and Proteinase Inhibitors. Fargo, ND (6/1/03)

BRMS1: Illuminating a surprising regulatory point for breast cancer metastasis? National Cancer Institute, Metastasis: Prevention or Therapy, Bethesda, MD (5/20/03)

Metastasis suppressor genes in human cancer, 18th Annual Symposium on the Biological Approaches to Cancer Treatment, Nagoya Japan (5/17/03)

A novel mechanism of metastasis regulation by the BRMS1 metastasis suppressor gene. University of Tokyo Symposium on Cancer Metastasis – Future Cancer therapy through understanding metastasis (5/16/03)

Genetics of breast cancer metastasis. Plenary Lecture, Era of Hope DOD Breast Cancer Research Program Meeting, Orlando, FL (9/26/02)

Do single cells constitute a metastatic lesion? Interactive Session – How can we keep metastatic lesions dormant?, Era of Hope DOD Breast Cancer Research Program Meeting, Orlando, FL (9/26/02)

Metastasis suppressor genes in human cancer: from discovery to mechanism of action to the clinic, MedImmune Inc. (7/17/03)

BRMS1: Biochemical advances, Lankaneau Research Institute Seminar (6/19/03)

Use of metastasis suppressor genes to prevent and treat metastasis. Eli Lilly Corporation (5/29/03)

A surprising mechanism for breast cancer metastasis suppression by BRMS1, Laboratory of Population Genetics, Center for Cancer Research (5/19/03)

Metastasis suppressor genes in human breast cancer. Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy (1/15/03)

Metastasis suppressor genes in human breast cancer. Penn State College of Medicine, Department of Pharmacology (11/18/03)

Metastasis suppressor genes: from discovery to mechanisms of action. M.D. Anderson Cancer Center, Division of Gastroenterology Seminar Series (10/24/02)

Metastasis suppressor genes: from discovery to mechanisms of action. Lombardi Cancer Center Tumor Biology Seminar Series (10/4/02)

Degrees obtained that were supported, in part, by this award

John F. Harms, Ph.D., degree granted May 2003. Dr. Harms also wrote and received an American Cancer Society Postdoctoral Fellowship.

Opportunities applied for and/or received based upon experience supported by this award

Since moving to UAB, I was asked to participate in the Breast SPORE grant based upon our experience with this DOD award. We have been funded for projects to explore breast cancer metastasis suppressor genes in bone metastasis in patient samples. A competing renewal, due October 2004, will evaluate clinical correlations of metastasis suppressors with incidence and, if possible, location of breast cancer metastasis.

Conclusions

We have made significant progress toward completing the aims of this proposal. Despite a slow start due to moving to UAB, graduation of John Harms, hiring and training of new personnel – Pushkar Phadke, Alexandra Silveira and Doug DiGirolamo – to carry out the experiments and a significant delay in official transfer of grant funds (did not occur until Spring 2004), we believe that we have made significant inroads toward a timely completion of the proposed aims. We have modified the statement of work slightly (to incorporate siRNA approaches), which was approved last year and anticipate that the modifications will allow us to be full-steam by mid- to late- summer 2004 with the new GFP-tagged variants.

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J Musculoskel Neuron Interact 2003; 3(1):30-38

Review Article

Breast cancer metastasis to bone: Evolving models and research challenges

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Overview of the clinical problem

When cancer is confined to breast, long-term survival rates are high. But, when cells metastasize, cure rates drop significantly (90% vs. 20% 5-year survival). Quality of life for patients with metastatic disease is also significantly worse than for patients with local carcinoma[1,2]. Thus, improvements in long-term survival will be most helped by better understanding of the metastatic process.

Skeletal metastases are common, particularly from breast, prostate and myeloma tumors. In many cases, the frequency of metastasis to bone is greater than metastases elsewhere. Whereas 73% of women develop bone metastases, only 33% develop lung and/or liver metastases. While patients can survive a relatively long time with bone lesions, their quality of life is miserable due to intractable pain, fractures, spinal cord compression and metabolic complications[3-6]. Besides the human cost, bone metastasis imposes a significant economic cost (2/3 of the costs of breast cancer treatment are due to bone metastasis[5]; ~$3 billion/yr[7]). The disparity between the clinical and economic importance of the problem and our knowledge of the underlying mechanisms responsible is staggering.

Nonetheless, there have been gains in knowledge regarding the mechanisms involved in breast cancer induction of osteolysis. This has led to improvements in treatment with drugs (e.g., bisphosphonates) designed to reduce loss of bone. Unfortunately, patients treated with these drugs seldom replace lost bone even when tumor cells are removed. Likewise, antecedent steps are largely understudied. In this review, we will focus on current knowledge about the earliest steps in breast cancer metastasis to bone. We will also present an evolving model for early steps of breast carcinoma metastasis to bone based upon currently available data and highlight some of the reasons for the relative sparsity of information about metastasis to bone.

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The metastatic cascade

Cancers derived from bone cells (e.g., osteosarcomas) are distinct from tumor cells that have immigrated to bone. Unfortunately, many lay people and even some physicians/researchers assume that bone-derived tumors are equivalent to bone-colonizing tumors. The reality is that the cell origins are different; the basal gene expression patterns are different and the underlying oncogenesis is different.

Metastasis is defined as the spread of tumor cells to establish a discontinuous secondary tumor mass. Tumor cells can get to other tissues by direct extension (not defined as a metastasis since the secondary lesion is not discontinuous from the primary tumor) or transport via blood vessels, lymphatics or in epithelial cavities. The predominance of metastatic spread to bone is thought to be via the hematogenous route.

Large numbers of tumor cells (in some cases $>10^7$ cells/day) enter the bloodstream daily, but fortunately establishment of secondary lesions is a rare event (i.e., $<<0.1\%$). In order to successfully form a metastatic colony, a specialized subset of tumor cells must possess all of the properties that give it selective survival and proliferative advantages over normal cells plus additional properties that confer the ability to spread and colonize secondary sites.

In the first step of metastasis, tumor cells must migrate away from the primary tumor and enter a circulatory compartment. Upon penetrating the basement membrane and endothelial barrier, tumor cells must evade innate immune surveillance and sheer mechanical forces associated with turbulent blood flow. At the secondary site, tumor cells either arrest because they are larger than the capillary diameter or they arrest because of tumor cell–endothelial recognition. After they have stopped moving, the cells must then divide in situ or extravasate. Extravasation requires the tumor cells to penetrate the intimal layer using a variety of motility and proteolytic mechanisms. Finally, tumor cells must proliferate in response to local growth factors and must be resistant to local growth inhibitors.

Development of metastasis contains stochastic elements as well as selection pressures. It is striking that breast cancer, prostate cancer and myeloma cells metastasize to bone 70-80% of the time[6]. The explanation for organotropism was first formally articulated by Sir Stephen Paget in his seminal paper in 1889[8]. In that work, Paget recognized that tumor cell <seed> and host <soil> properties worked in concert to determine success of metastasis. Rather than a comprehensive review of the literature, we will focus on the extravasation steps and terminal tumor cell–bone cell interactions that determine the osteolytic process.

Besides predisposition of cancer cells to colonize bone, it is crucial to understand that not all bones are equally involved. The predominance of osseous metastases occur in the long bones, ribs or vertebrae[6]. Furthermore, the metastases tend to occur at the ends of the bones, near the trabecular metaphyses. Therefore, it is essential to understand what is special about the trabecular bone structure and environment that make it amenable to frequent colonization.

Properties of the bone microenvironment that contribute to metastasis

The metaphyseal region is characterized by a meshwork of trabecular bone, rich blood flow and red bone marrow. Interdigitating the trabecular tongues are bone marrow in close proximity to the vascular sinusoids. The vascular and marrow compartments are separated by a trilamellar structure composed of endothelium, basement membrane and supportive adventitial cells[9]. Trabecular bone is covered by osteoblasts and bone lining cells; the latter are believed to differentiate into osteoblasts. Bone lining cells and osteoblasts have many properties in common, including alkaline phosphatase and Type I collagen expression[10].

Metastatic breast carcinoma cells that arrive in the metaphyses first interact with sinusoidal endothelial cells that line the vascular system. Binding probably occurs in a manner similar to leukocyte homing[11]. Compared to other tissue sites, it is less likely that tumor cell arrest in bone is non-specific. Rather than a network of small diameter (e.g., 5-10 μm) capillaries in the lungs or sinusoids of the liver (~30 μm), the diameters of the sinusoidal lumens can be several hundred microns in diameter.

Blood flow in sinusoids is also amenable to tumor cell arrest. Blood flow in sinusoids is sluggish compared to capillaries and post-capillary venules[12,13]. In murine calvaria, where blood cells can be readily visualized, blood flow in the venous sinusoids is ~30-fold lower than the arterial rate[12]. Schnitzer et al. measured blood flow using microsphere distribution in canine long bones and found that flow in metaphyseal and marrow cavities was 7-14 ml/min/100 gm tissue, compared to ~200 ml/min/100 gm tissue in post-prandial intestine[14].

Taken together, these properties suggest that more specific recognition properties are involved in tumor cell homing to bone. Among the more appealing hypotheses related to bone organotropism are the endothelial "addresses". A growing body of evidence suggests that lymphocytes and tumor cells can recognize unique macromolecules or combinations or surface molecules on bone endothelium[15,16].

In contrast to vascular endothelium elsewhere in the body, bone endothelial cells simultaneously and constitutively express the tethering molecules, p-selectin and e-selectin, and vascular cell adhesion molecules, VCAM-1 and ICAM-1[12,17,18]. In other cells, expression is transient in response to inflammatory stimuli[11,19]. In light of findings that metastases are more frequent at sites of inflammation[20-22], it is intriguing to speculate that tumor cells bind well to sinusoidal endothelium because those cells have similar surface markers as cells at an inflammatory site. The hypothesis gains credence because many breast carcinoma cells express the counter-receptors for these ligands[23-25].

Histological examination of bone metastases shows tumor cells in intimate contact with bony surfaces. It follows, then, that tumor cells penetrate the endothelial barrier or extravasate. Cancer cells in close proximity to vascular endothelial surfaces have been shown to stimulate endothelial cell retraction[26]. For example, osteonectin secretion by breast cancer cells has been reported to stimulate flux of macromolecules and pulmonary endothelial cell rounding[27]. HER2/neu over-expressing MCF-7 cells have been shown to stimulate vascular endothelial cell retraction[28].

Extravasation is, by definition, a directional movement. Therefore, it follows that tumor cells may be responding to bone-derived chemotactic gradients. Several examples consistent with this hypothesis have been observed. Three molecules that are highly expressed in bone – osteonectin, osteopontin, bone sialoprotein, collagen – have been shown to be chemoattractants for some tumor cells[29-32].

Osteonectin, which is produced by osteoblasts, has recently been shown to be a powerful chemoattractant for several prostate cancer cell lines and one breast cancer cell line[29,33]. Moreover, osteonectin can increase endothelial monolayer permeability[27] and has been shown to induce matrix metalloproteinase-2 secretion by MDA-MB-231 breast carcinoma cells[34,35].

Osteopontin is produced by many cell types, including osteoblasts, breast epithelium, breast and other types of can-

cer cells. In bone, osteopontin is deposited in matrix, binds to hydroxyapatite and serves as an anchor for osteoclast binding via the avb3 integrin[36]. Breast carcinoma cells also frequently express the high affinity avb3 integrin. As bone resorption occurs, Ca^{++}, PO_4 ions and matrix proteins are released. It is possible that intact and fragmented forms of osteopontin serve as diffusible chemotactic factors for breast cancer cells. In breast cancer, osteopontin is secreted in a soluble form[37]. Metastatic MDA-MB-435 cells have been shown to migrate toward soluble osteopontin fragments[30]. In addition to this limited list, osteopontin has been shown to be a promoter of metastasis in a variety of other systems (reviewed in[38,39]).

Bone sialoprotein is secreted primarily by osteoblasts[40,41] fosters chemotactic migration via an RGD-dependent binding to avb3 integrin[31]. Like the other matrix-derived proteins described above, it has multiple roles in both normal bone tissue and in the development of skeletal malignancies.

Chemokines are a family of small, cytokine-like peptides that induce cytoskeletal rearrangement, adhesion to endothelial cells and directed cell migration[42-44] and are therefore ideal for serving in the metastatic process. This notion was recently elegantly confirmed by Taichman et al.[45] who, considering the fact that hematopoietic cells use osteoblast-derived CXCL12/SDF-1 to home to bone normally, examined this factor in prostate cancers. They found that all bone metastases from prostate cancers expressed the CXCR4 receptor for SDF-1 and that SDF-1 increased prostate cancer cell migration and adherence *in vivo*. Muller et al.[46] cataloged expression of known chemokine receptors and found that breast cancer cell lines express abundant CXCR4 and/or CXCR7. This finding was particularly enlightening since the ligands for CXCR4 and CXCR7 are CXCL12/SDF-1 and CXCL21/6Ckine, respectively. The ligand expression is most abundant in tissues to which breast cancers most frequently metastasize (bone marrow, lymph node, lung and liver) and less abundant in less frequently involved tissues (intestine, kidney, skin, brain, skeletal muscle). They hypothesized that a combination of chemotactic factors present in bone matrix (e.g., CXCL12, osteonectin, osteopontin and others) could interact with a repertoire of receptors on breast cancer cells that confer the high specificity of these cancers for the skeleton.

Finally, once breast carcinoma cells have made their way into bone, many find the growth environment particularly hospitable. The precise molecular basis for breast cancer growth in bone is not known, but it is easy to speculate that the microenvironment is rich in growth factors based upon the normal function of bone marrow for sustaining stem cells and hematopoiesis. Indeed, the milieu of the bone marrow is ideal for many proliferating cells. Additionally, the continuous remodeling of the bone matrix would contribute to the growth potentiating surroundings by release of matrix-bound factors.

Thus, metaphyseal bone appears to have a unique combination of properties that renders it highly attractive to certain cancer cells. These properties include: a) slowed blood flow which may allow time for cell–cell interactions to occur; b) large lumenal diameters which would reduce sheer; c) constitutively expressed array of vascular surface proteins that may contribute to initial cancer cells binding; d) expression of matrix-associated molecules and chemokines which could serve as potent chemoattractants for tumor cells; and e) a milieu of growth factors which would provide a rich environment for tumor cell proliferation.

Entry of tumor cells into the bone microenvironment disrupts homeostasis

Bone matrix is constantly undergoing reorganization, based upon an intricate ballet of matrix-depositing cells (osteoblasts) and matrix–degrading cells (osteoclasts). When tumor cells enter the trabecular-marrow space, the balance is disrupted. In most breast cancers, the balance is shifted toward net bone degradation. It is beyond the scope of this review to discuss the many mechanisms involved in bone turnover and readers are referred to several outstanding reviews on this topic[47-50].

While many factors regulate bone turnover, members of the tumor necrosis family (TNF) and TNF receptor families appear to be essential. RANK-Ligand (receptor activator of nuclear factor kappa B, NFkB, ligand) is a TNF family member expressed by stromal cells and osteoblasts while RANK is expressed by osteoclasts; however, it was not detected in breast cancer cells[51]. *In vivo* and *in vitro* evidence indicates that interaction of these two molecules is essential for osteoclastogenesis. Other factors (e.g., glucocorticoids, vitamin D3, IL-1, IL-6, IL-11, IL-17, TNF-α, PGE2, PTH, and PTHrP) may modulate expression levels.

Osteoprotegerin (OPG, also known as osteoclastogenesis inhibiting factor) is another osteoblast-derived product that counters bone loss caused by RANK-L/RANK interactions[48,49]. OPG can serve as a decoy receptor for RANK-L. Interestingly OPG can also bind and inactivate TRAIL (TNF-regulated apoptosis-inducing ligand) and prevent TRAIL-initiated osteoblast apoptosis[52]. Under normal conditions OPG balances bone loss by competing with RANK-L for RANK on osteoclasts. However, OPG expression is down-regulated by breast cancer cells[53].

The RANK-L/RANK/OPG system may also explain how chronic inflammation and autoimmune diseases can cause bone loss. Activated T cells express RANK-L and also produce pro-inflammatory cytokines, e.g., TNF-α, IL-1, IL-11, IL-6 which up-regulate RANK or Fas or other death molecules in osteoblasts[54]. T cells also produce IFN- (which suppresses bone loss). In addition, activated macrophages secrete many of the same pro-inflammatory cytokines as the stromal cells. Thus, the inflammation associated with the presence of metastatic tumor cells favors bone loss. A current model in the literature presents these three molecules, RANK-L, RANK and OPG, as the basic factors controlling normal skeletal remodeling[47]. Other factors modulate the

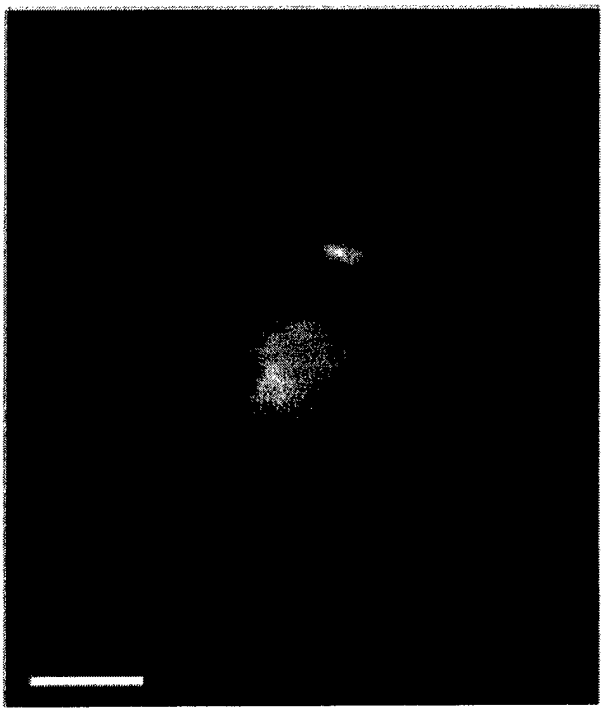

Figure 1. Representative image of whole bone with GFP-tagged tumor cells. Three separate lesions are visualized using GFP. The uppermost lesion contains elements that are brighter than the majority of cells. Frequently, this is indicative of full or partial penetration of tumor cells penetration through the bone. Bar = 1 mm.

system indirectly by up-regulating or down-regulating RANK-L, RANK and OPG. One of these regulatory molecules is PTHrP.

PTHrP (parathyroid hormone related peptide) is produced in excess by many metastatic cancer cells. Its effects were known long before the molecule was identified. Early in the twentieth century a connection was made between hypercalcemia and neoplastic diseases. The next 70 or so years were spent trying to explain this association and to discover how hypercalcemia associated with metastasis was different from that seen in hyperparathyroidism. It is now known that the molecule critical in metastatic hypercalcemia is PTHrP. The N-terminus of PTHrP is structurally homologous to parathyroid hormone (PTH) and has PTH-like activity although it is a product of a different gene. PTHrP binds to a G-protein-coupled receptor on osteoblasts[55]. Thus, PTHrP acts on osteoblasts to indirectly cause bone resorption mediated by osteoclasts. PTHrP produced locally in excess by metastatic tumor cells can bind to PTH/PTHrP receptors on osteoblasts and cause them to up-regulate RANK-L and down-regulate OPG[48,53]. The result is the differentiation of preosteoclasts and the activation of mature osteoclasts to become fully bone resorbing cells. This activity can be further enhanced by TGF-β which is released as the bone matrix is resorbed. While TGF-β has normally been shown to down-regulate RANK-L expression by osteoblasts and thus decrease resorption[56], many metastatic breast cancer cells express TGF-β receptors. TGF-β binding to the receptor induces PTHrP production[57]. Thus, a so-called "vicious cycle" is established in which osteolytic metastasis indirectly enhances osteoclastogenesis[47] and provides a positive feedback loop. Recent reports by Gay et al.[58] and Faucheux[59] and earlier reports (reviewed by Gay and Weber[60]) show that osteoclasts also have PTHrP receptors, suggesting a direct action of PTHrP on osteoclasts even if osteoblasts are absent.

In short, tumor cells manipulate the bone microenvironment upon entering the metaphyseal region. While tumor cells themselves can cause bone matrix resorption[61,62], the predominant mechanism is usurping the mechanisms used in normal bone physiology. As noted above, the predominance of research into the mechanisms of breast cancer-induced osteolysis have focused on activation of the osteoclast. However, another mechanism could also be operative, inactivation or elimination of the osteoblast.

Normally, osteoclasts remain viable for 2-3 weeks, whereas osteoblasts exist for 2-3 months or more[63]. If the lifespan of osteoclasts were increased or the lifespan of osteoblasts decreased, the net effect would be bone loss because the basic bone unit (osteoblast: osteoclast ratio) would be out of balance. Detailed studies of proliferation and apoptosis in these cells has not been extensively studied; however, we have obtained evidence that osteolysis-inducing breast tumor cells can increase apoptosis of osteoblasts[64]. This observation is consistent with the clinical observations that osteolytic lesions often have fewer osteoblasts and that patients treated with osteoclast-inhibiting bisphosphonates do not normally repair the bone defects (i.e., because they no longer have sufficient viable osteoblasts in the region)[62,65]. Clearly, additional studies are needed in this area.

Models to study skeletal metastasis in breast cancer

Although metastasis to bone is a common and serious problem, it has historically been extremely difficult to study. In large part, this is due to the near-complete lack of experimental models that recapitulate the metastatic process. An ideal model would replicate the entire metastatic cascade (i.e., growth of a primary tumor to metastasis). However, there are currently no human cancer cell lines that reproducibly metastasize to the bone from an orthotopic site, (i.e., mammary gland)[66]. There is only one rodent model that spreads from an orthotopic site to bone (4T1[67]). While 4T1 is an important model, worldwide experience with it has not been sufficient to ascertain whether it is predictive of biology in humans. Recently, several transgenic mouse models have been developed which exhibit metastatic capacity[68-75]. However, to the best of our knowledge, none of them metastasize to bone.

An alternative methodology for studying bone metastasis

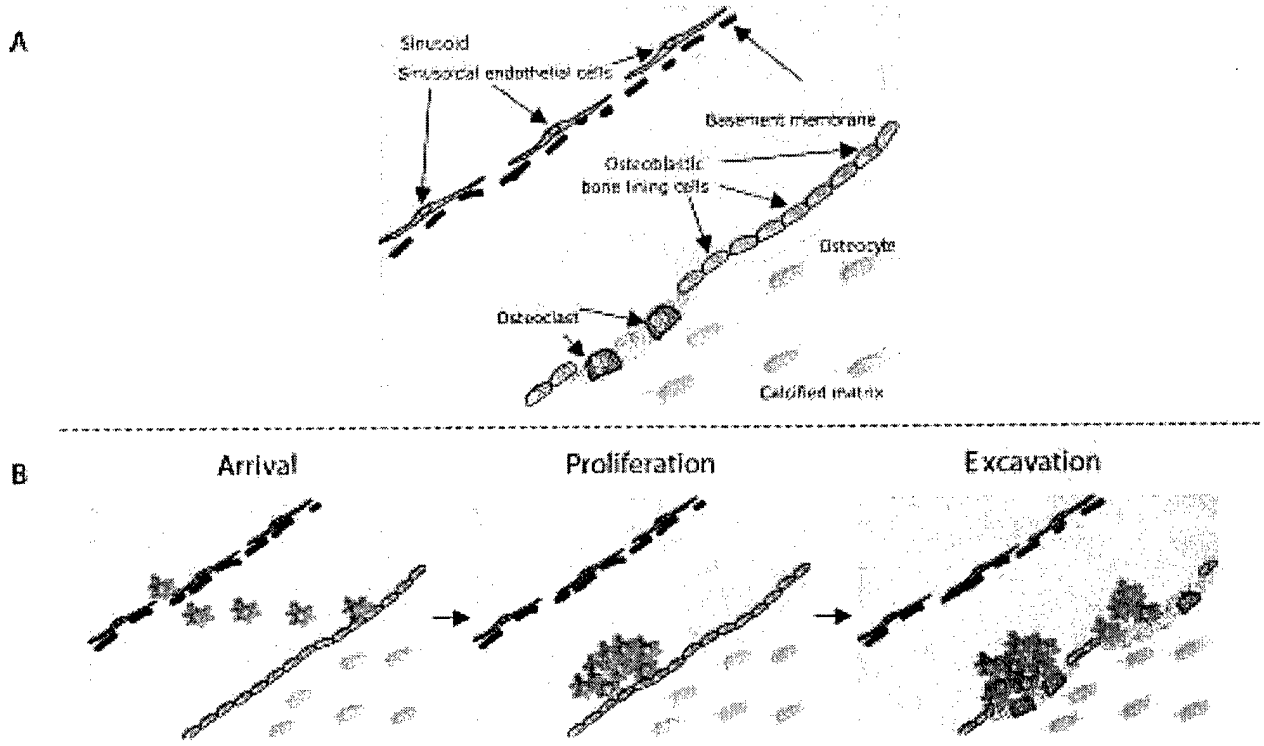

Figure 2. Schematic diagram of trabecular bone with the major cell types highlighted (A). Panel B represents the three major steps of bone metastasis formation. Tumor cells arrive in the bone via the vascular sinusoids and bind to the specialized endothelium. After the tumor cells pass through the endothelial barrier and extravasate through the underlying basement membrane, they migrate toward the trabecular bone surface which is lined by osteoblastic bone lining cells. Tumor cells then proliferate in response to local growth factors. Breast cancer cells that enter the bone disrupt the balance between osteoblast and osteoclast activities, resulting in a net bone loss. Osteolysis (excavation) can be accomplished by tumor cell: (i) activation of osteoclasts; (ii) inactivation of osteoblasts; (iii) a combination of osteoclast activation and osteoblast inactivation; or (iv) direct tumor cell degradation of bone matrix.

was pioneered by Arguello[76], who injected melanoma cells into the left ventricle of the heart. Yoneda and colleagues adapted this procedure using MDA-MB-231 human breast cancer cells and showed reliable colonization of bone with subsequent osteolysis[77,78]. The bulwark of the field and the vast majority of experimental data in the breast field with regard to bone metastasis have been collected using this cell line. We recently showed that another human breast carcinoma cell line, MDA-MB-435 could also form osteolytic lesions following intracardiac injection[79]. Yoneda, Guise and colleagues have shown that MCF7 and T47D variants can form osteoblastic metastases following intracardiac injection as well[51].

Besides the inherent limitation of extrapolating findings using limited numbers of cell lines, the experiments with bone metastasis were limited by technology as well. Basically, the standard method for detecting bone lesions – radiography – requires ≥50% bone degradation to be detectable. This means that only the latest stages of bone colonization and osteolysis can be studied. Histological examination is arduous and time-consuming. Serial sectioning of bone is technically challenging; so, step sections are more commonplace. As a result, small lesions can be easily missed. Again, studying early steps of bone colonization are not well-served by this technique.

To alleviate some of these limitations, we engineered MDA-MB-435 and MDA-MB-231 cells to constitutively express enhanced green fluorescent protein (GFP). This modification has increased detection sensitivity tremendously[79]. Representative images are depicted in Figure 1. GFP-expressing cancer cells can be detected through the intact bone even when radiographic evidence of tumor involvement is not apparent. We have even been able to detect single GFP-tagged cancer cells in bone. Furthermore, GFP allows three-dimensional examination and the ability to distinguish foci visually. This technique offers the capability of studying metastasis early in the process, before major bone degradation has occurred. The stages beginning with microscopic metastasis and latency, and ending in aggressive bone degradation can now be separated. Moreover, the response of the bone cells including osteoblasts, ranging from bone lining to fully differentiated cells, as well as osteoclasts can

be examined before they are destroyed as part of metastatic tumor growth.

The genetics of cancer cell metastasis to bone

We have been interested in determining the underlying genetic defects responsible for cancer metastasis. Specifically, our laboratory has identified metastasis suppressor genes for human breast carcinoma[80-83] and melanoma[84-87]. Data with the metastasis suppressor for melanoma is instructive to the discussion of organotropism.

Late-stage melanomas have losses or rearrangements of the long-arm of chromosome 6 in 66-75% of cases. Since losses occurred concomitant with acquisition of metastatic potential, we hypothesized that a metastasis suppressor gene was encoded on 6q. To test this, we introduced an intact copy of chromosome 6 into a metastatic human melanoma cell line[87]. The resulting hybrids were completely suppressed for metastasis while primary tumor growth still occurred. Subsequent experiments showed that the chromosome 6 – melanoma cell hybrids were able to complete every step of the metastatic cascade, except proliferation at the secondary site[88]. Recovery of single cells in lung followed by injection into the skin (i.e., the orthotopic site) showed that the cells grew well[88], suggesting that the metastasis suppressor gene(s) were organ specific. To evaluate this possibility, we injected chromosome 6 – melanoma hybrids into the left ventricle of the heart and monitored metastasis to all organs (J.F. Harms and D.R. Welch, manuscript in preparation). Metastasis was suppressed to all organs except bone.

While our results are striking, they are not completely unprecedented. Rinker-Schaeffer[89-91] and Steeg[92] have shown that the metastasis suppressor genes MKK4 and Nm23 also inhibit at late stages of the metastatic cascade. Additionally, using intravital microscopy, Chambers, Groom and colleagues have described frequent arrest and extravasation of tumor cells without subsequent proliferation at the secondary site[93,94]. Our results extend those findings to demonstrate (we believe for the first time) organ-specific metastasis suppression. The implication is that there will be classes of genes that determine organotropism of metastasis. On a theoretical level, this is not surprising. However, while the seed and soil hypothesis has been around for over a century, this is among the first molecular footholds into understanding the mechanism(s) responsible.

Working model for the earliest steps of bone metastasis

The simplest model for bone metastasis formation involves three steps. **Arrival:** Tumor cells enter bone through the vasculature, adhering strongly and preferentially to metaphyseal region sinusoidal endothelium and/or basement membrane. **Proliferation:** Tumor cells then migrate into the bone marrow space and eventually proliferate to form macroscopic lesions. [Note: the mere presence of single tumor cells does not constitute a metastasis which, by definition, is a tumor mass.] It is not entirely clear whether proliferation precedes osteolysis since the latter may release growth stimulatory signals from the matrix. **Excavation/Osteolysis:** Tumor cells interact with trabecular, osteoblast-like bone-lining cells, osteoblasts and osteoclasts to initiate the cascade of events leading to matrix dissolution.

Each of the steps of bone metastasis involves the interplay between breast carcinoma cells and bone cells. Understanding how the bone cells and tumor cells communicate will be essential to controlling metastasis to bone. Recently, we found human breast carcinoma cells that were suppressed by transfection of the metastasis suppressor gene BRMS1 exhibited restored homotypic gap junctional intercellular communication[95,96]. Studies are underway to explore whether there are differences between metastasis-competent and metastasis-suppressed cells with regard to heterotypic communication.

Conclusions

Metastasis to bone is an important clinical problem that has been relatively understudied. Recent development of models has provided, for the first time, the opportunity to study the earliest steps of the process of bone colonization. Careful utilization of the new models and expansion of the number of available models will provide new insights into the initial events taking place during bone colonization.

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MDA-MB-435 human breast carcinoma metastasis to bone

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Abstract

Breast cancer metastasizes to bone with high frequency and incidence. However, studies of breast cancer metastasis to bone have been limited by two factors. First, the number of models that colonize bone are limited. Second, detection of bone metastases is too insensitive or too laborious for routine, large-scale studies or for studying the earliest steps in bone colonization. To partially alleviate these problems, the highly metastatic MDA-MB-435 (435) human breast carcinoma cell line was engineered to constitutively express enhanced green fluorescent protein (GFP). While 435^{GFP} cells did not form femoral metastases following orthotopic or intravenous injections, they produced widespread osteolytic skeletal metastases following injection into the left ventricle of the heart. All mice developed at least one femur metastasis as well as a mandibular metastasis. As in humans, osseous metastases localized predominantly to trabecular regions, especially proximal and distal femur, proximal tibia, proximal humerus and lumbar vertebrae. 435^{GFP} cells also developed metastases in adrenal glands, brain and ovary following intracardiac injection, suggesting that this model may also be useful for studying organotropism to other tissues as well. Additionally, GFP-tagging permitted detection of single cells and microscopic metastases in bone at early time points following arrival and at stages of proliferation prior to coalescence of individual metastases.

Abbreviations: 231 – MDA-MB-231; 435 – MDA-MB-435; CMF-DPBS – calcium- and magnesium-free Dulbecco's phosphate-buffered saline solution; FACS – fluorescence activated cell sorting; GFP – enhanced green fluorescent protein

Introduction

Breast cancer directly affects one in eight women [1]. Of women who develop breast cancer, as many as 85% will develop metastases in bone [2]. Skeletal colonization by breast cancer cells most frequently causes osteolytic lesions with corresponding sequelae – pathological fractures, spinal chord compression, pain and hypercalcemia. Despite its prevalence, studies of breast cancer metastasis to bone are infrequent, limited by a paucity of models and the technical challenges associated with detection of osseous metastases. Thus far, research of breast cancer metastasis to bone has been predominated by a single human cell line (MDA-MB-231 [3–7]) and recently, a murine cell line (4T1 [8, 9]). In most cases, studies have focused on late stages of bone metastases (i.e., osteolysis) because analysis of early steps (e.g., tumor cell arrival and colonization) has been infeasible.

Despite being uniformly derived from metastases, surprisingly few human breast carcinoma cell lines retain the capacity for metastasis in immune-compromised mice. Even fewer metastasize efficiently from the orthotopic site [10, 11]. Research of breast cancer metastasis has been dominated by two human breast carcinoma cell lines, MDA-MB-231 (231) and MDA-MB-435 (435), but recently, additional lines are being developed [12, 13]. Bone metastasis research has hinged almost exclusively upon 231 [4, 5, 7, 14, 15], with isolated studies using other cell lines [12, 13, 16, 17]. Although there are sporadic claims to the contrary [18], colonization of bone by 231 cells requires injection into the left ventricle of the heart. And while 435 cells can grow in bone if directly injected [16], the ability to colonize bone has heretofore not been systematically examined.

Studying metastasis to bone requires methods to routinely detect bone lesions. Because they are located in a solid matrix, bone metastases are readily visible only when considerable red marrow is displaced. In the absence of a pigment, colorimetric marker or bioluminescent tag, identification of skeletal metastases depends upon laborious histological sectioning or radiographic detection. Radiography requires sufficient (e.g., > 50% [19]) osteolytic reduction in bone mass; so, microscopic metastases confined within the marrow are overlooked entirely. Detection of microscopic metastases by histology is technically feasible but tedious and impractical for large-scale studies. These limitations have been partly alleviated by detection of B16 melanoma metastases in bone because of endogenous melanin production [20]. Others have used cells tagged with β-galactosidase

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(lacZ) [21–23] or luciferase [24]. Unfortunately, additional cofactors are necessary to detect these reporters. In contrast, the convenience and utility of fluorescent molecules, such as enhanced green fluorescent protein (GFP), for the detection of metastases has been clearly demonstrated in many sites [25–27], including bone [18, 28, 29].

In this report, we compare 435 metastasis to bone following orthotopic, intravenous and intracardiac injection. In addition, we take advantage of the increased sensitivity of GFP detection to map the distribution of microscopic and macroscopic skeletal metastases.

Materials and methods

Cell lines and culture

Metastatic human breast carcinoma cell line, MDA-MB-435 (435) was a generous gift from Dr Janet E. Price (University of Texas–M.D. Anderson Cancer Center, Houston) and was stably transfected with pEGFP-N1 (BD Biosciences Clontech, Palo Alto, California) by electroporation (Bio-Rad Model GenePulser^{TM}, Hercules, California; 220 V, 960 μFd, $\infty\Omega$). Neomycin resistant cells were selected for growth in, and maintained in, a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/F-12; Invitrogen, Gaithersburg, Maryland), supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.02 mM non-essential amino acids, 5% fetal bovine serum (Atlanta Biologicals, Norcross, Georgia) and 500 μg/ml Geneticin (G418; Invitrogen). The brightest 25% of fluorescing cells were sorted using a Coulter EPICS V cell sorter (Beckman-Coulter, Fullerton, California). All cultures were confirmed negative for *Mycoplasma* spp. infection using a PCR-based test (TaKaRa, Shiga, Japan).

In vivo *metastasis assays*

Immediately prior to injection, cells at 80–90% confluence were detached from 100-mm cell culture plates (Corning, Acton, Massachusetts) with 2 mM EDTA and 0.125% trypsin in calcium- and magnesium-free Dulbecco's phosphate-buffered saline solution (CMF-DPBS). Cells were counted using a hemacytometer, and resuspended in Hank's balanced salt solution to the appropriate final concentration. For spontaneous metastasis assays, cells (1×10^6 in 0.1 ml) were injected into the right subaxillary mammary fat pad of anesthetized (ketamine-HCl 129 mg/kg, xylazine 4 mg/kg) 5–6 week-old female athymic mice (Harlan Sprague-Dawley, Indianapolis). Food and water were provided *ad libitum*. Resulting tumors were removed at a group mean tumor diameter [11] of 12 mm and mice were necropsied four weeks later. Lungs and femurs were removed and viewed by fluorescence microscopy (see below) prior to fixation. Macroscopic lung metastases, were also quantified as described [11].

For intravenous (i.v.) and intracardiac (i.c.) injections, cells (2×10^5 in 0.2 ml) were injected into 4–5-week-old female athymic mice via the lateral tail vein or left ventricle of the heart, respectively, using a 27 gg needle and 1 ml tuberculin syringe. Intracardially injected mice were fully anesthetized. Immediately preceding and subsequent to inoculation, drawback of bright red arterial blood into the syringe was used as an indication of arterial administration, as opposed to darker, burgundy colored blood. Mice were necropsied four or five weeks post-injection. Distribution of bone metastases was mapped following examination of all thoracic and abdominal organs. Bones were dissected free of musculature and soft tissues using a #21 scalpel blade and gauze or squares of paper towel to grip and remove remnants. Where possible, bones were left connected (e.g., femur–tibia–fibula, scapula–humerus–radius–ulna, ribcage–vertebrae) to facilitate orientation. Following external fluorescence examination of the dissected skull for bone and brain metastases, a sagittal bisection of the skull was performed to expose the brain interior.

Animals were maintained under the guidelines of the National Institute of Health and the Pennsylvania State University College of Medicine. All protocols were approved and monitored by the Institutional Animal Care and Use Committee.

Fluorescence microscopy

To visualize metastases derived from the GFP-tagged cell line, intact viscera and whole bones (dissected free of soft tissue), were placed into petri dishes containing CMF-DPBS and examined by fluorescence microscopy utilizing a Leica MZFLIII dissecting microscope with 0.5× and PlanApo 1.6× objectives and GFP fluorescence filters ($\lambda_{excitation}$ = 480 ± 20 nm, $\lambda_{emission}$, 510 nm barrier) (Leica, Deerfield, Illinois). Photomicrographs were collected using a MagnaFire^{TM} digital camera (Optronics, Goleta, California), and ImagePro Plus software (Media Cybernetics, Silver Spring, Maryland).

Faxitron X-ray analysis

Dissected bones were X-rayed using a Hewlett-Packard Faxitron model 43855B and Kodak X-Omat TL film (Kodak, Rochester, New York). Tube voltage was set at either 19 kVp or 59 kVp, and exposure time was determined automatically.

Bone decalcification and storage

Intact, dissected bones from individual mice were placed in 25-ml glass scintillation vials and fixed in freshly prepared 4% paraformaldehyde in CMF-DPBS at 4 °C for 24–48 h. Bones destined for histological sectioning were subsequently removed and decalcified in 0.5 M EDTA in CMF-DPBS for 18–24 h before paraffin embedding. Non-embedded bones could be stored long-term (months) at 4 °C with retention of fluorescence if the solution was replaced at 1–5 days with 0.5 M EDTA in CMF-DPBS or 1% paraformadehyde in CMF-DPBS. Fluorescence was typically lost if tissues were stored in 4% paraformaldehyde or ethanol solutions.

Mapping of bone metastases

During fluorescence microscopy, skeletal metastases were drawn on diagrams of murine bones (adapted from [30]). A custom computer program was written using Visual Basic 6 (Microsoft Corp., Redmond, Washington) in which the same diagrams were overlaid with a grid of squares (~ 0.30 mm^2). Metastases drawn for each mouse bone were transferred to the computerized grid. The program then calculated the percentage of mice in which tumor encompassed each square in the grid and depicted a composite image using color or grayscale. Composite images were then smoothed in Photoshop 6.0 (Adobe, San Jose, California) to reduce granularity.

Results

Skeletal metastases obtained via intracardiac injection

MDA-MB-435 cells were transfected with a plasmid conveying enhanced GFP under a cytomegalovirus constitutive promoter. The resulting mixed population of neomycin resistant cells contained both fluorescing and non-fluorescing clones. Cells comprising the highest 25% of fluorescence intensity were selected using a fluorescence activated cell sorter. Cells (1×10^6) were injected into the mammary fat pad of female athymic mice. Tumorigenicity and *in vivo* growth rates of the resulting 435^{GFP} tumors were indistinguishable from the parental line (data not shown). Pulmonary metastatic potentials were likewise not significantly different. Only a small fraction of 435^{GFP} cells lost or had decreased fluorescence when continuously cultured. Nonetheless, to validate fluorescence as a method to quantify metastases, lungs were fixed in Bouin's solution following fluorescence microscopy and macroscopic metastases recounted. The number of lung metastases using fluorescence and traditional methods was nearly identical in most cases ($n = 11$), differing by only 1 to 3 metastases. In only one mouse were counts significantly greater following Bouin's staining (36 vs.15 metastases), suggesting outgrowth of non-fluorescing clones. Thus, the number of metastases numerated under fluorescence would represent, at worst, an under-estimation. In subsequent fluorescent analyses, steps were taken to monitor for non-fluorescent skeletal metastases.

Metastatic potential of 435^{GFP} cells was assessed following orthotopic, i.v., or i.c. injection in a pilot experiment. The objective was primarily to evaluate bone metastasis formation. While it has been previously shown that 435 cells infrequently establish pulmonary metastases following i.v. injection [31], bone colonization following this route had not been reported. To minimize first-pass clearance of cells in the lung microvasculature (the first capillary bed encountered by cells entering the venous circulation), 435^{GFP} cells were injected i.c. Four weeks following tumor removal or vascular injection, mice were necropsied; both femurs removed, dissected free of soft tissue and scrutinized by fluorescence microscopy. Femoral lesions did not develop following injection into the mammary fat pad or i.v. inoculation (Figure 1). Green fluorescent foci were observed in the femurs of intracardially injected mice with high frequency. Moreover, the metastases were osteolytic (Figure 2A). In mice necropsied following longer durations, osteolytic lesions were apparent by radiography (Figures 2B, C).

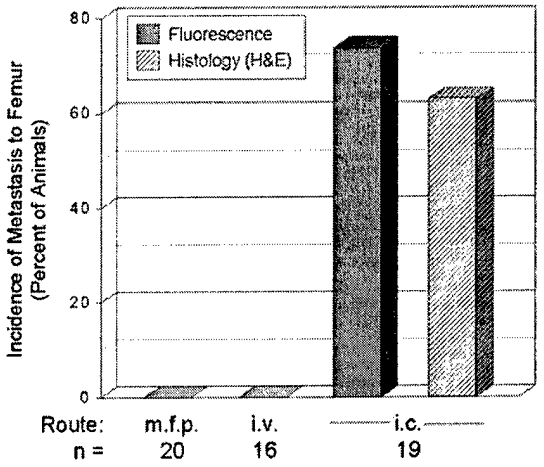

Figure 1. Skeletal metastases are obtained following intracardiac injection of 435^{GFP}, but not following orthotopic (mammary fat pad) or intravenous injection. The number of detectable metastases to bone is increased by GFP-tagging (compared to step section analysis of bones), although the increase is not significant. Cells (2×10^5) were introduced into 4–6-week-old female athymic mice by intracardiac injection. Mice were necropsied at four weeks and femoral bones dissected free of soft tissues. Femurs were first examined by fluorescence and then using H&E-stained sections at five levels.

GFP-tagging allows detection of bone metastases

To determine whether the convenience of GFP detection translates to increased detection of macroscopic metastases in bone, femurs of intracardially injected mice were fixed in 10% neutral buffered formalin, decalcified and embedded in paraffin for standard histology. Longitudinal sections representing five levels through approximately two-thirds of the bone were stained with hematoxylin and eosin. Tumor in histological sections corresponded to green fluorescence observed in 435^{GFP} injected mice. However, fluorescent foci were detected in two mice that were undetected in the limited number of sections evaluated. Incidence of 435^{GFP} bone metastases by histology from 63% (1.7 ± 0.37; mean $\pm$ SEM), compared to 74% (2.4 ± 0.46) by fluorescence (Figure 1). The difference was not statistically significant and could be explained by sampling error in the histology. Additionally, in this pilot analysis, mice with bone metastases had multiple lesions in each femur. This made us question whether mice with no metastases were successfully injected in the left ventricle. For subsequent studies, the color of blood drawn into the syringe was assessed prior to, and after, injection. When arterial injection was verified in this manner, incidence of bone metastasis increased to 100% of femurs.

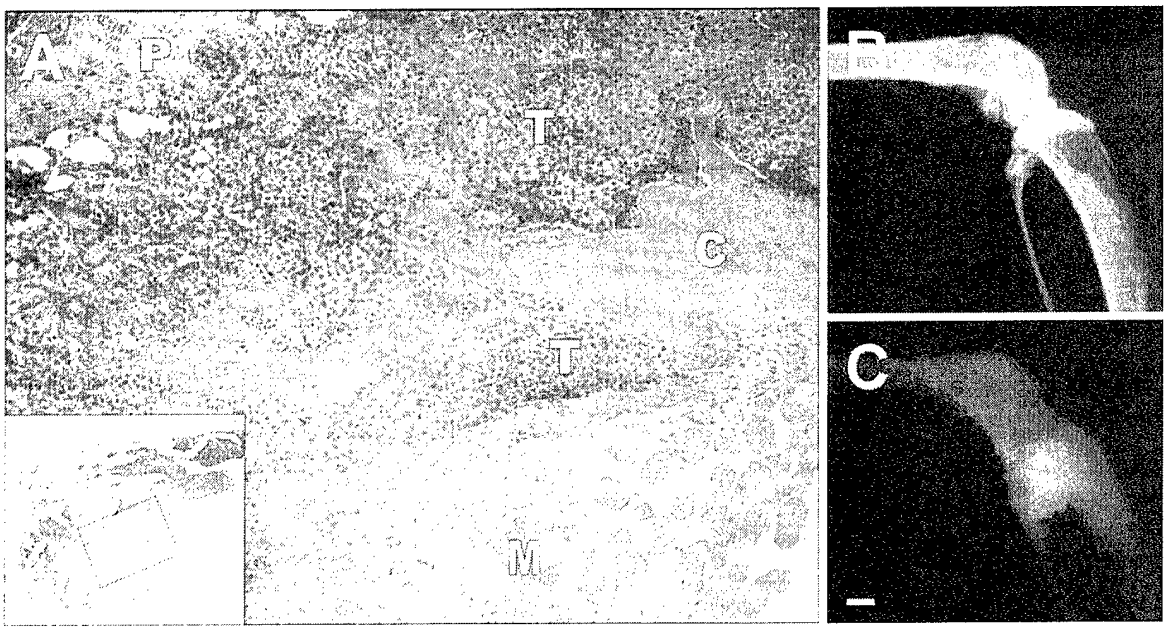

Figure 2. 435^{GFP} skeletal metastases are osteolytic. A. A metastasis is shown in distal femur four weeks following intracardiac injection. Tumor within the medullary cavity has invaded through the cortical bone to the exterior of the shaft. Cortical bone (C), tumor (T), distal epiphyseal growth plate (P), skeletal muscle (M). B. X-ray (19 kVp) 7.5 weeks following i.c. injection shows significant osteolysis in proximal tibia corresponding to a fluorescing lesion (Figure C). Bar = 1 mm.

To determine whether skeletal metastases were randomly distributed, 435^{GFP} cells were injected i.c. and all bones (femur, tibia, fibula, scapula, humerus, radius, ulna, pelvis, skull, mandible, ribcage, vertebrae) were examined five weeks later by fluorescence. Bones were examined following removal of soft tissues. While not essential for detecting large metastases in the vertebral column or exposed joints such as the knee, detection of microscopic lesions and deep joints (e.g., proximal femur) required dissection of musculature. 435^{GFP} produced skeletal metastases with highest incidence in femur and mandible (Figure 3A). While 100% of mice ($n = 16$) had at least one femoral metastasis, 56% of mice had involvement of both femurs. Overall, 78% of femurs had at least one metastasis. Mandible metastases were found in all mice (94% of bones, 2 dentary bones per mandible). Sixty-three percent of mice developed vertebral metastases, accounting for 13% of all cervical, thoracic, lumbar and sacral vertebrae examined. Skull, pelvis, humerus and tibia were also involved in $\geq 50\%$ of animals. Except for the vertebral column, which yielded a mean of 4 ± 1.6 (mean $\pm$ SEM) metastases per mouse, the greatest number of metastases per mouse were in femur (2 ± 0.3) and mandible (2 ± 0.2) (Figure 3C).

The location and size of fluorescent metastases were graphed and the distribution of metastases was evaluated using custom software. As in humans, metastases localized predominantly to trabeculae in appendicular bones (proximal and distal femur, proximal tibia, and proximal humerus (Figure 4A)). Within the vertebral column, the lumbar and sacral vertebrae were involved with higher incidence than cervical or thoracic vertebrae.

Metastasis to non-osseous sites

Viscera and other organs were also evaluated for metastases. Except for brain, in which a sagittal bisection was performed, fluorescent metastases were quantified in intact tissues (Figures 4B–D). Using a relatively simple setup allows visualization of metastases within most tissues [32]. The most frequent sites of non-skeletal metastasis included adrenal glands (11/16 mice), brain (8/16) and ovary (7/16). Few macroscopic pulmonary metastases were observed (3/16), limited to only 1–3 macroscopic metastases per mouse. Numerous microscopic metastases (1–10 cells) were present in a total of 8 mice. Metastases also developed in the pancreas, kidney, liver, and eye in three to five mice. Rare metastases were encountered in stomach, uterus, bladder and spleen. Mice (4/16) also had metastases in mesenteric lymph nodes, but the number involved per mouse ranged from 11 to > 70.

GFP allows assessment of early time points in bone colonization

Tracking the arrival of metastasizing cells and subsequent proliferation at the secondary site has revealed key information regarding the role of the microenvironment in metastasis [25, 33, 34]. To assess the effectiveness of GFP tagging in the detection of metastases in bone at early time points, mice were necropsied following intracardiac injection of 435^{GFP} cells, beginning at 10 min. Single fluorescing cells were seldom detectable in intact femur; however, longitudinal bisection revealed single cells in the bone interior (Figure 4B). At two weeks, microscopic metastases and single cells were

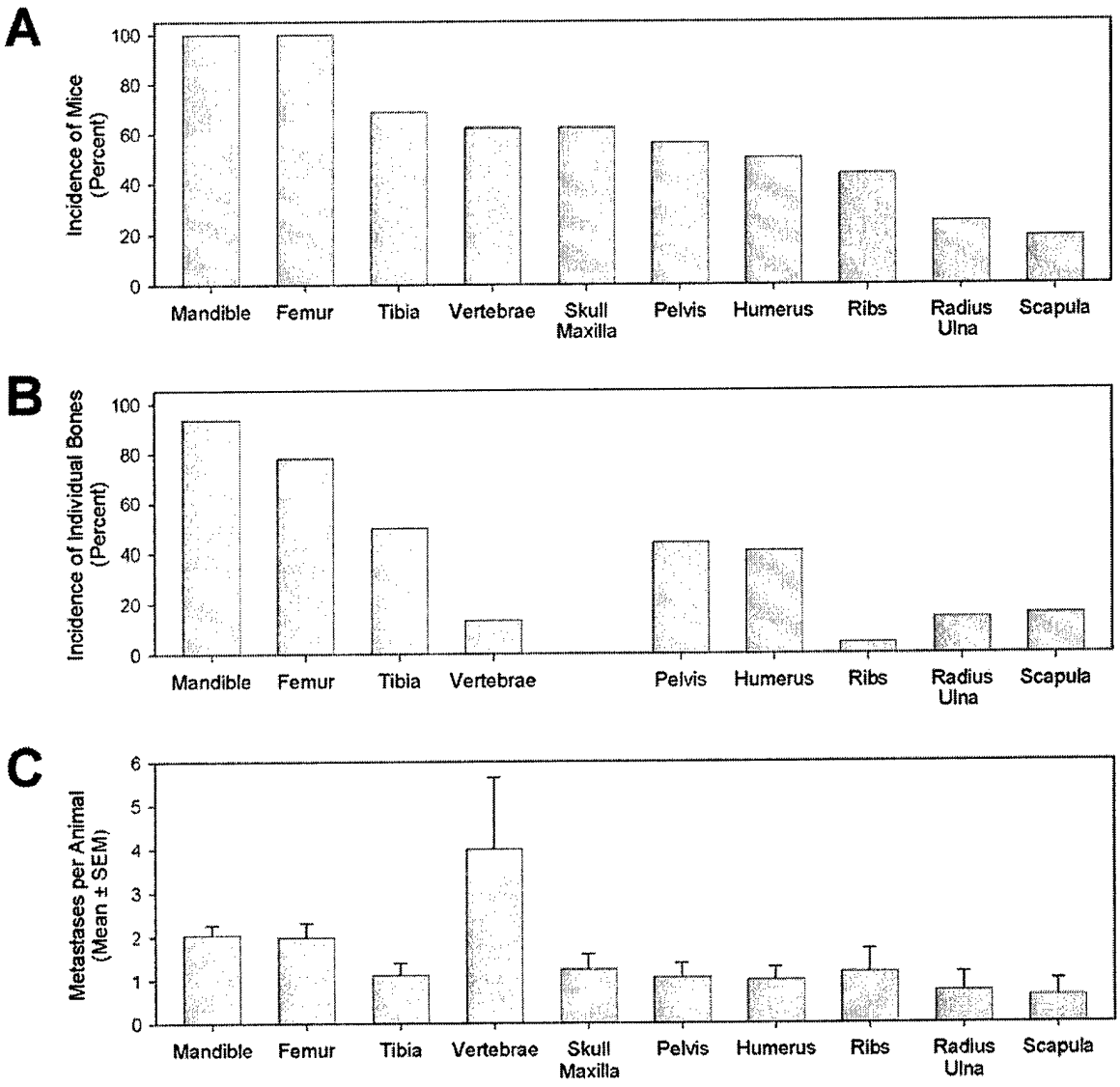

Figure 3. Distribution of skeletal metastases. Mice necropsied 5 weeks after injection of 2×10^5 435^{GFP} cells into the left ventricle. Intact bones were dissected free of soft tissue and examined by fluorescence microscopy. A. Percent of mice with at least one metastasis in specified bones (skull and facial bones are grouped) ($n = 16$). B. Percent of individual bones with at least one metastasis. (mandible considered as 2 bones; pelvis, 2; ribs, 26; vertebrae, 30). C. Number (mean ± SEM) of metastases per mouse within specified bones ($n = 16$).

observed through uncut bone (Figures 4D, E). Observation of GFP through intact bone permitted convenient three-dimensional examination of lesions because bones could be fully rotated and manipulated. Adjacent, but separate foci could be distinguished prior to coalescing (Figure 4). In addition, macroscopic metastases were readily detected prior to radiographic evidence of osteolysis (Figure 5).

Discussion

The human breast carcinoma cell line, MDA-MB-435, has been widely used in the study of human breast cancer, both *in vivo* and *in vitro*. It has been extremely useful because it is one of the few breast cancer cell lines that metastasizes. However, its propensity to colonize bone, the most common site of breast cancer metastasis, has not been thoroughly examined. Previously, a single study directly injected 435 cells into bone [16]; however, lesions formed by this method cannot be construed as metastases.

To assess whether 435 might be a useful model for bone metastasis, we stably transfected cells with enhanced green fluorescent protein. The resulting cells behaved as parental cells in tumorigenicity and spontaneous metastasis assays (i.e., following orthotopic injection). As we have observed previously, GFP does not appear to adversely affect tumor cell behavior. GFP transfection was performed in

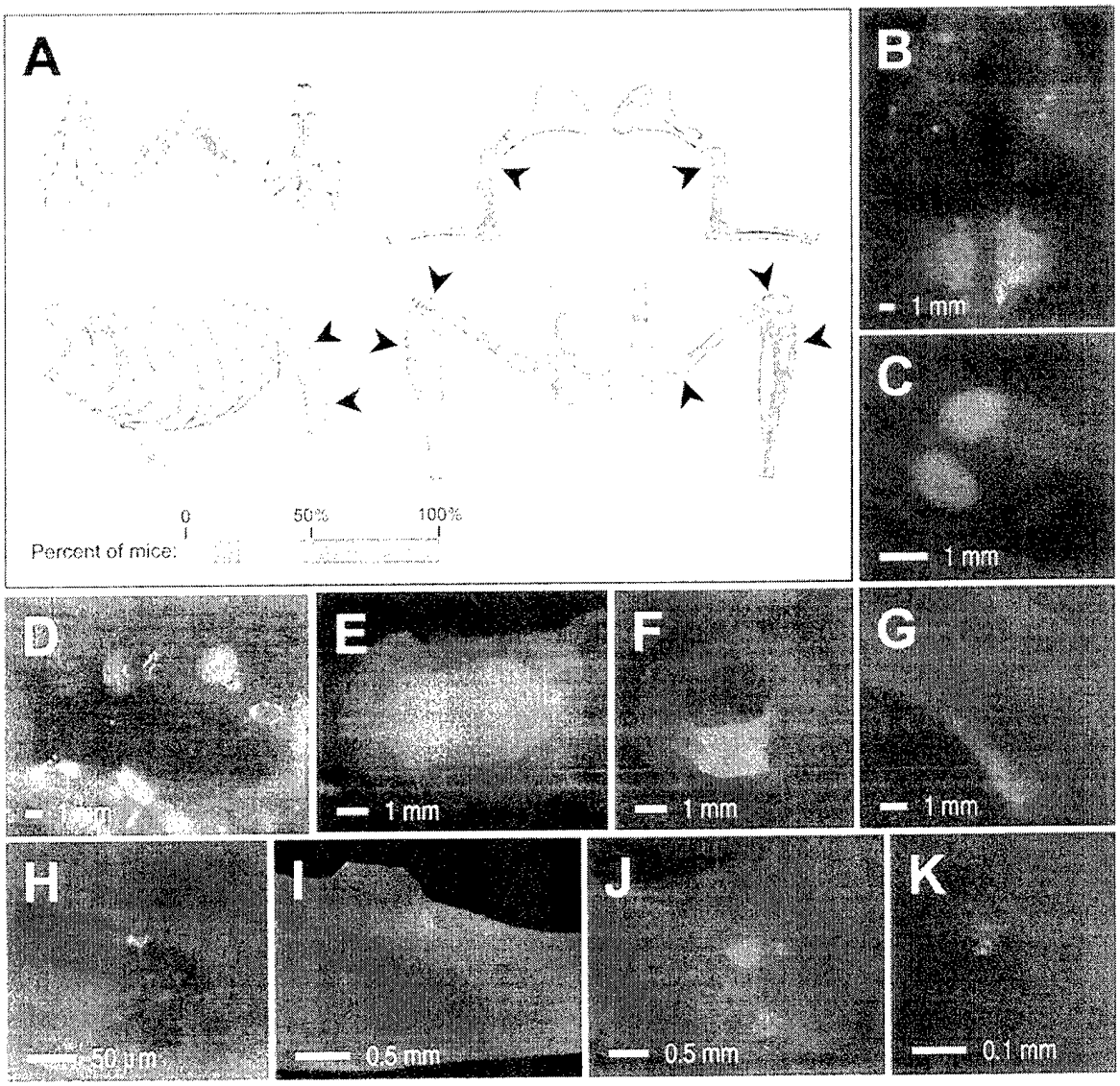

Figure 4. A. Compilation of 435^{GFP} skeletal metastases in 16 mice. Metastases (five weeks) localize predominantly to trabecular regions in femurs, proximal tibia, proximal humerus and vertebrae. Mandibular metastases are frequent. Arrowheads highlight regions of the skeleton with the highest incidence of bone metastases. B. Sagittal bisection of the skull and brain reveals a fairly large number of 435^{GFP} brain metastases 5 weeks following i.c. injection. C. Metastases (5–6) in adrenal gland. D. Rare 435^{GFP} metastases to liver. E. Two involved lumbar vertebrae at four weeks. F. The metastasis in the left most vertebrae of (E) is localized to the centrum. G. A scapular metastasis. H. Single tumor cells and microscopic metastases are detectable by fluorescence microscopy. Longitudinal bisection revealing single 435^{GFP} cell in distal femur 10 min following intracardiac injection. Note, the cell is already forming pseudopodial processes. I. Left tibia two weeks post-intracardiac injection. Cluster of three to five cells visible from the exterior of intact bone. J. Whole, distal left femur two weeks post-intracardiac injection. Multiple adjacent, but separate, foci are easily distinguished. By four to five weeks, such lesions would most likely have coalesced. K. Three 435^{GFP} cells visible at the lung surface two weeks following intracardiac injection.

order to enhance the detection of tumor cells in the bone. Our data show that fluorescent detection was greater than radiographic methods or step-sections through bone. Bone metastases formed at sites similar to those colonized in breast cancer patients (proximal appendicular long bones, vertebrae, pelvis) [35, 36]. In patients, 80–90% of skeletal metastases occur in the axial skeleton [35, 37], whereas we observed 62% of metastases are within axial bones in our model. The pattern and frequency of metastasis following intracardiac injection of 435^{GFP} cells was similar to those for the widely used 231 cells [3, 5, 14, 18, 38–41]. Additionally, Sasaki et al. [41, 42] have used 231 to study maxillofacial bone metastases, which typically comprise approximately 1% of oral malignancies. So, the high frequency of 435 mandibular involvement suggests this would be a suitable model for this metastasis site as well. Further supporting the quality of the 435^{GFP} model, metastases were predominantly osteolytic, as in the majority of human breast cancers. Also, the majority of bone lesions occurred in metaphyseal tra-

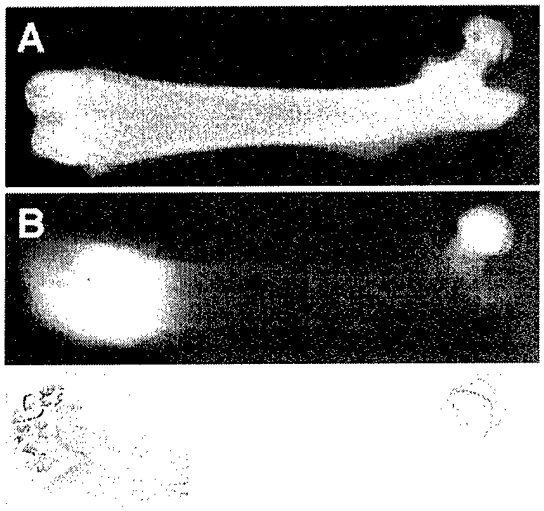

Figure 5. Metastases, visible by fluorescence microscopy (B) 4 weeks following i.c. injection, are not detectable by radiography (59 kVp) (A). H&E stained histology confirms the presence of tumor (C).

beculae, sites most commonly colonized in human cancer metastasis to bone.

In addition to osseous metastases, 435^{GFP} cells colonize several other organs that are frequent sites of breast cancer metastases – adrenal gland, brain and ovary. Rare lesions were found in lung, pancreas, kidney, liver and eye. Thus, the intracardiac injection model using 435^{GFP} affords opportunities to study metastases of human breast carcinoma to other relevant sites in a xenograft model.

From a technical perspective, this report highlights several issues. First, extrapulmonary metastases are infrequent unless 435^{GFP} cells are injected into arterial circulation. Proper injection into the left ventricle of the heart could be routinely validated by careful examination of blood color prior to and after tumor cell inoculation. The additional manipulation did not appear to have any adverse effect on the mice. Viability and complete recovery within 30 min were routine. Second, GFP allowed detection of metastases in intact bones. Building on the pioneering work of Hoffman and colleagues, who examined melanoma, prostate and lung cancer metastases to bone [28, 29, 32, 43], we developed the 435^{GFP} breast carcinoma model. During the course of these studies, Peyuchaud et al. reported development of 231^{GFP} variants [18]. GFP-tagging allowed detection of lesions one week prior to radiographic detection. We were similarly able to detect single cells or microscopic foci within two weeks, almost two to four weeks prior to radiographic evidence of osteolytic metastases. The ability to detect metastases before severe osteolysis provides a powerful tool for studying the earliest stages of bone colonization. In addition, the potential to minimize pain and suffering associated with more extensive bone involvement (e.g., paralysis or fracture) provides significant ethical improvement. Additionally, obviating the need for histology to observe bone metastases is a major savings in time and resources. Third, the sensitivity of GFP detection permits imaging of single cells. While we had previously used fluorescently labeled tumor cells to quantify single cells in lung, the capacity to detect microscopic, single cell foci within intact bone or in a bone which had been bisected was a fortuitous finding. Coupled with newly developed techniques that allow decalcification and sectioning, while maintaining fluorescence [44], we believe that it is now possible to study the earliest steps of tumor cell arrival and movement within the bone micro-environment. Fourth, we were able to store tissues for long periods (several months) while maintaining fluorescence. This ability provides investigators with adequate time to thoroughly examine tissues in large scale experiments involving multiple experimental groups.

In conclusion, we have added another human breast carcinoma cell line to the armamentarium for studies of metastasis to bone. By incorporating improved detection due to fluorescent tagging, a model is now available for studying the earliest steps in osseous metastasis and for large scale experiments where significant osteolysis is not desirable.

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Genomic analysis of primary tumors does not address the prevalence of metastatic cells in the population

Ramaswamy *et al.*[1] compared gene expression profiles of adenocarcinoma metastases to unmatched primary adenocarcinomas. They found an expression pattern that distinguished primary tumors from metastases but also reported that a subset of primary tumors had the expression pattern of metastases. This finding led them to challenge "the notion that metastases arise from rare cells within the primary tumor"[1]. In fact, their finding provides no evidence to contradict this notion.

To produce a metastasis, a tumor cell must complete a series of sequential steps, including detachment, invasion, survival in the circulation, attachment, extravasation, proliferation, induction of neovasculature and evasion of host defenses[2]. Because metastases are largely clonal in origin[3–5], the successful metastatic cell must have a set of characteristics that enable it to complete each step in the sequence. Lack of any single characteristic derails the process and prevents the cell from developing into a metastasis. Thus, the successful metastatic cell has been likened to a decathlon champion, who must be proficient in all ten events, not just a few, to be successful[2]. A primary tumor may contain many different cells, each of which can complete some of the steps in the metastatic process but not all. In aggregate, all of the steps may be represented among cells of the primary tumor, but it may still be the rare cell that can complete all the steps and thus give rise to a metastasis. The study by Ramaswamy *et al.*[1] looked at primary tumors in aggregate and, therefore, cannot rule out this possibility. The authors seem to have overlooked the large body of evidence indicating that primary tumors are heterogeneous with respect to many characteristics, including those associated with metastasis[2,6,7]. One example came from our work in which we found, by cloning, that unselected tumor cell lines with low metastatic potential contained a small number of cells with high metastatic potential, as well as many non-metastatic cells[3]. More recently, *in situ* hybridization was used to detect the expression of genes associated with the metastatic phenotype, specifically, those encoding MMP-2, MMP-9 and E-cadherin[8–10]. This approach allows not only the detection of gene expression but also its visualization in the tumor. These studies showed that expression of these three genes varied independently between the peripheral and central zones of the tumor and among other regions in a single section of the tumor. It stands to reason that the more cells express such genes, the higher the likelihood will be that the tumor will eventually give rise to metastases, a correlation substantiated in retrospective studies[9,10]. The findings of Ramaswamy *et al.*[1] using a genomics approach are consistent with those using *in situ* hybridization but have the added advantage of being able to identify previously unknown genes involved in the metastatic process.

Much evidence supports the view that progression from a benign to a malignant tumor is associated with acquisition of a set of genetic and epigenetic alterations that provide the phenotypic characteristics of malignancy[11–13]. These changes accumulate at different rates in different tumors and are reflected, albeit imperfectly, in the pathologist's classification of tumor stages. The stage I and II lung adenocarcinomas and early breast cancers studied by Ramaswamy *et al.*[1] generally expressed the non-metastatic pattern of genes, and only a few expressed the metastatic pattern. This probably reflects the fact that some of these primary tumors have indeed generated unique cells with full metastatic capabilities, as indicated by the patient survival data. The true significance of the study of Ramaswamy *et al.*[1] is not that it runs contrary to popular dogma, which, in our opinion, it does not, but that it may enable the identification of the small subset of tumors designated as early stage by pathologic criteria that nonetheless have already released a few metastatic cells. Thus, the study constitutes an important step in the quest to predict the behavior of tumors detected at an early stage, even though it does not address the prevalence of fully metastatic cells in primary tumors.

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Genetic background is an important determinant of metastatic potential

Recently there has been some debate about the etiology of cancer metastatic potential. Using microarray gene expression patterns of breast carcinomas, van't Veer *et al.*[1] reported that a set of 117 genes predicted metastatic potential. More recently, a small set of 17 genes was reported to predict metastatic potential for a variety of solid tumors[2]. These findings suggest that most primary tumor cells express a 'metastasis signature', in contrast to the classic model, which predicts that only a rare subpopulation of primary tumor cells have accumulated the numerous alterations required for metastasis. Based on this evidence, Bernards and Weinberg[3] recently posited that combinations of early oncogenic alterations, not specific events that promote metastasis, determine metastatic potential. This hypothesis might explain why metastasis occurs in some individuals with small, localized tumors (that is, tumors whose

cell number is too small to have statistical likelihood of accumulating adequate numbers of mutations proposed in the conventional model).

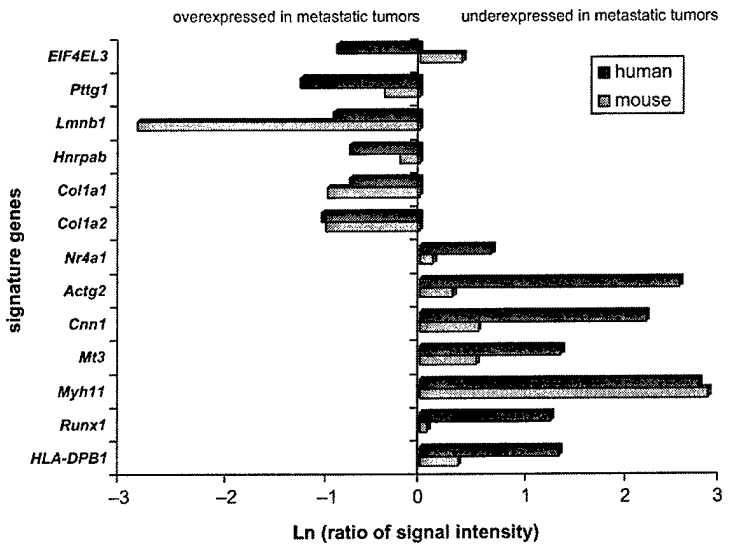
In contrast, there is persuasive evidence for the existence of mutations that promote metastasis. For example, metastasis-specific loss of heterozygosity has been associated with many solid tumors. Based on the tumor-suppressor paradigm, several laboratories have cloned genes that, when reintroduced into tumor cells, suppress the formation of secondary tumors without altering primary tumor initiation or kinetics. So far, eight metastasis-suppressor genes have been described (reviewed in ref. 4).

Thus, compelling evidence for both models exists. How, then, can these seemingly conflicting hypotheses be reconciled? One possibility, based on our studies, would be the contribution of genetic background. Using a transgene-induced mouse tumor model and a breeding strategy to vary genetic background, we found significant differences in metastatic efficiency (as much as 10-fold) between the original FVB/NJ mice and F1 hybrids without altering tumor initiation or growth kinetics[5,6]. We recently examined microarray data from our high-efficiency and low-efficiency metastatic genotypes for the set of 17 genes that comprise the metastasis signature[2]. Of these 17 genes, 13 were represented on the mouse chip. The expression of 12 of these changed in the same direction as in the human set (see figure).

Because all tumors were initiated by the same oncogenic event, differences in the metastasis microarray signature and metastatic potential are probably due to genetic background effects rather than different combinations of oncogenic mutations. Consistent with our observations in metastasis, several laboratories have shown similar strain differences with regard to oncogenesis, aging and fertility in transgenic mouse models[7–9]. Data on both primary tumors and metastases reinforce the notion that tumorigenesis and metastasis are complex phenotypes involving both inherent genetic components and cellular responses to extrinsic stimuli.

Thus, although our expression data is preliminary and additional studies are required to confirm these results, the cumulative data suggest that differential gene expression patterns may reflect individual genetic profiles that, in turn, are important determinants of metastatic potential. Unlike highly penetrant cancer susceptibility genes, metastasis susceptibility is probably due to complex allelic combinations. Work in our laboratories has shown that multiple genes probably affect the efficiency of this process[6].

The metastatic paradox may, therefore, by resolved by combining the two hypotheses: metastatic potential is determined early in oncogenesis but primarily by host genetic background (rather than oncogenic mutations), on which specific mutations that promote metastasis then occur. The theory also suggests that some families may be more susceptible to metastasis. If this were carried to its logical extension, the data imply that it might be possible to define metastasis susceptibility based on gene expression in readily accessible tissues (for example, blood) rather than from tumor. This would be a less costly and less invasive method to predict metastatic propensity.



Comparison of gene expression profiles in the mouse and human metastasis signature sets. Gene expression is represented as natural log of the signal intensity ratio either of human primary to human secondary metastases or of mouse low-efficiency to high-efficiency metastatic genotypes. Genes overexpressed in metastatic tumors fall to the left of the center line; those underexpressed fall to the right.

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Mini Review

Metastasis suppressor pathways—an evolving paradigm

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Abstract

A greater understanding of the processes of tumor invasion and metastasis, the principal cause of death in cancer patients, is essential to determine newer therapeutic targets. Metastasis suppressor genes, by definition, suppress metastasis without affecting tumorigenicity and, hence, present attractive targets as prognostic or therapeutic markers. This short review focuses on those twelve metastasis suppressor genes for which functional data exist. We also outline newly identified genes that bear promising traits of having metastasis suppressor activity, but for which functional data have not been completed. We also summarize the biochemical mechanism(s) of action (where known), and present a working model assembling potential metastasis suppression pathways.

Keywords: Metastasis; Suppression; Genes; Cancer; Tumor; Tumorigenesis; KISS1; MKK4; BRMS1; CRSP3; TXNIP; E-cadherin; CRMP1; Maspin; CD44; SSeCKS; Nm23; KAI1; TIMPs; DRG-1; Metastin; Multigene; Invasion; Prognosis; Therapeutic; Marker; Target; Anoikis; Proliferation; Apoptosis; Angiogenesis; Neovascularisation

1. Introduction

Despite better local treatments for cancer using surgery and radiotherapy, the clinical challenge remains combating systemic metastatic disease. Metastasis via the lymphatics, hematogenous system, or through the body cavities results in significant morbidity. Not only must cells leave the primary tumor, but they must also proliferate at the secondary site [1,2]. Metastasis culminates the evolution of tumor cells whereby a tumor's composition collectively becomes progressively more malignant [3,4]. Tumor progression results from genetic instability coupled with selection of subpopulations of cells [3]. Eventually some cells accumulate sufficient capacity to dissociate and spread. Depending on whether the mutations occur early or late in tumor progression determines proportions of metastatic cells within tumors of a given size. This conclusion can be appreciated when interpreted in light of classical studies of Luria and Delbrück [5]. Selection of metastatic cells varies with the nature of a tumor as well as between patients. Although it is generally true that larger tumors are more likely to spread, size does

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not necessarily correlate with metastatic capacity [6,7]. In addition to accumulating mutations, there are exogenous signals that can influence metastatic efficiency.

2. Host–tumor interactions in neoplastic advancement

Tumorigenicity and metastasis are distinct, but interrelated phenotypes. Tumorigenicity is necessary, but not sufficient, for metastasis. In part, metastasis is also determined, to a great extent, by tumor–host interactions. That is, the microenvironment participates in the induction and selective proliferation of malignant cells [8].

How does the host environment at the metastatic site affect the metastatic behavior of cells? The relationship is reciprocal, and reflects both host endocrine and immunologic status. Host physiology can foster or reject neoplastic cells. In response to tumor-secreted cytokines and chemokines, diverse leukocyte populations are recruited including neutrophils, dendritic cells, macrophages, eosinophils, mast cells and lymphocytes. All inflammatory cells can produce a plethora of cytokines, proteases (e.g. MMPs), membrane-perforating agents and soluble cytotoxic mediators (e.g. TNF-α, interleukins and interferons) [9]. For example, tumor-associated macrophages, play a dual role in tumor development. They can kill neoplastic cells following activation by IL-2, IL-12 and interferons; but they can also induce angiogenesis by growth factor, cytokine and proteinase secretion [9]. Indeed proteinases in the tumor milieu are largely stroma-derived [10]. Thus, metastatic tumor cells can modify the host environment so that tumor cells are nurtured.

Tumor–host interactions formed the basis of Sir Steven Paget's 'seed and soil' theory [11] to explain the predilection of breast cancer spread to bone. He proposed that the tumor cells (seed) are scattered in many directions by the circulatory system, but grow only in response to the microenvironments of specific organs (soil). While this review focuses on metastasis genes (i.e. in the seed), we emphasize that the regulation of those genes by the host cannot be ignored. That is, the context in which the genes function must be considered, even though the details are not yet known.

3. Stochastic and selective aspects of cancer metastasis

In order to metastasize, cells must complete a series of sequential steps, each of which is rate-limiting. Following primary tumor growth (including establishment of neovasculature or primitive vascular channels [12,13]), tumor cells detach and enter a circulatory compartment. The tumor vasculature is immature and incontinent [14], providing easier access to the vasculature. Once there, tumor cells can remain as single cells or form homo- or heterotypic emboli but they must survive shear forces as well. At the secondary site, tumor cells can arrest due to size restriction or become tethered to vascular endothelium using a variety of surface adhesion molecules. In some cases, tumor cells recognize endothelial addressins—surface molecules that designate the cells as from a particular organ, tissue or vessel structure [15–18]. Additionally, tumor cells can respond to chemoattractants produced by different tissues [9,19]. For the most part, the identity of the attractants are not yet known [20], but recent data implicate chemokines [9, 21–23]. Depending upon tumor type and the tissue in which the tumor cells have arrested, cells can begin to proliferate within the vasculature or extravasate before proliferating [24–28]. Merely getting to the secondary site does not constitute a metastasis. Metastases are defined as secondary *masses*.

Overall, the process of metastasis is quite inefficient [29,30]. Cells in the vasculature are cleared biphasically [29,31]. The initial phase (6–24 h), represents an exponential decline of cell number, presumably due to mechanical trauma, oxygen toxicity, anoikis and immune clearance. A second, more gradual decline, presumably represents cell death at secondary sites [29]. Tumor cells that arrive at a second site do not necessarily proliferate immediately. Some cells may remain 'dormant' for extended periods or until conditions become favorable for proliferation [32–35].

Dormancy of pre-angiogenic metastases is more accurately described as a balance between

proliferation and apoptosis [36]. Wong et al. [37] found that the majority of cells underwent apoptosis within 24 h of intravasation. If apoptosis was inhibited, metastatic potential increased. In contrast, Luzzi et al. [33], and Cameron et al. [38] found that most cells survived, but failed to proliferate. It is not yet possible to reconcile these two apparently conflicting conclusions. However, since the tumor cells and host tissue were not identical and since the data are not mutually exclusive, it is likely that both are correct. It is probable that the rate-limiting steps of metastasis will vary by cell lines and in different tissues, reflecting yet another level of heterogeneity within tumors.

Technical advances have made it possible to detect single cancer cells or microscopic foci in experimental models [39–42]. If model data are extrapolated to the clinical setting, diagnosis and treatment decisions become significantly more complex. The issue is whether microscopic foci justify aggressive treatment because of their potential to grow into overt lesions. Or, if the percentage of cells that eventually proliferate is vanishingly small, should patients be spared toxic chemotherapy since the mere detection of cell clusters at a secondary site does not necessarily translate into establishment of macroscopic metastases?

Considerations such as these underscore the need for markers that can be used to accurately and definitively predict metastatic potential (in this case, defined as the possibility of forming macroscopic metastases) [43]. New technologies such as microdissection, microarray, real-time RT-PCR, proteomics and comparative genomic hybridization (CGH) are being evaluated to define and characterize metastatic potential of cancer specimens [44–53]. Identifying molecules that are specifically involved in metastasis (as opposed to indirect changes in gene expression due to tumor progression) presents a daunting challenge as well as significant opportunity. The difficulty relates to discriminating between mere association from causality [2,43,54–57]. Metastasis suppressor genes are attractive candidates for marker development because, by definition, their loss should be associated with the acquisition of metastatic potential [58]. Moreover, they represent potential therapeutic targets.

We emphasize that, while it takes a finely orchestrated set of functions to metastasize, blockage of even one step halts the process. Since the discovery of the first metastasis suppressor gene, *nm23*, more than a decade ago, the number of metastasis suppressors identified has grown significantly (reviewed in Ref. [2]).

Various studies involving CGH, loss of heterozygosity (LOH) and karyotype analysis identified distinctively altered regions and/or genomic imbalances involving various human chromosomes [55]. Some changes correlated temporally with acquisition of metastatic propensity. By inference, then, those chromosomal regions were thought to predict the location(s) for metastasis-associated genes. In the case of genetic loss, replacement of the chromosomes by microcell-mediated transfer (MMCT) was predicted to suppress metastasis. MMCT has been instrumental in identifying several metastasis suppressor genes.

MMCT of chromosomes 2, 7, 8, 10, 11, 12, 13, 16, 17 and 20 suppressed metastasis of prostate carcinoma cells without blocking tumorigenicity (reviewed in Ref. [59]). By positional cloning regions on chromosome 17 were narrowed to an ~70 cM [60]. Yoshida et al. [34] eventually cloned the MKK4 metastasis suppressor gene. Details regarding individual genes will be provided below. The identities of the invasion-suppressing genes with regard to metastasis suppression have not been as easily forthcoming. Importantly, inhibition of invasion (unless *completely* inhibited) does not necessarily suppress metastasis. While invasion is required for metastasis, tumor cells must merely be able to accomplish the step [43,56,61,62]. They do not have to be extraordinarily efficient at component processes.

Structural alterations involving chromosome 6 are frequent in metastatic melanoma [63]. MMCT of full-length human chromosome 6 suppressed metastasis of the human metastatic melanoma cell line C8161 [64,65]. Chromosome 6 hybrids were less motile, but just as invasive [66]. Chromosome 6 hybrids engineered to express green fluorescent protein were used to demonstrate that they completed every step of the metastatic cascade except proliferation at the secondary site [67]. Using subtractive hybridization the *KISS-1* metastasis suppressor was identified [68]. Also using the C8161 melanoma, MMCT of chromosome 1 suppressed metastasis [69].

Alterations of chromosome 11 in metastatic breast carcinoma are well documented [51]. Following

MMCT of chromosome 11 into the metastatic human breast carcinoma cell line, MDA-MB-435, hybrids were significantly suppressed for lung and lymph node metastasis [70].

MMCT has been the most lucrative technique for identifying metastasis suppressors. However, other approaches (subtractive hybridization, differential display and microarrays) have been used successfully and their frequency of identification is rapidly growing.

4. NM23

By screening cDNA libraries of matched metastatic/non-metastatic K1735 murine melanoma cell lines by differential hybridization, 'non-metastatic clone 23' gene, was identified as the first metastasis suppressor gene [71]. Enforced expression in cell lines of diverse cellular origin, suppressed metastasis without altering tumor growth (reviewed in Ref. [72]). The product of the human ortholog, *NM23-H1*, was identified to be a nucleoside diphosphate kinase (NDPK). NDPKs catalyze the transphosphorylation of the γ-phosphate of a deoxynucleoside triphosphate to a deoxynucleoside diphosphate with the formation of a histidine-phosphorylated intermediate. The *Drosophila* nm23 ortholog, *awd*, is required for proper differentiation of tissues of epithelial origin (reviewed in Ref. [73]). To date, eight NM23 family members have been identified, designated *NME1* through *NME8*. Of these, *NM23-H1* and *NM23-H2* have reported metastasis suppressor activity, but NDPK activity has been dissociated from metastasis suppression [74]. Postel and colleagues identified Nm23-H2 as a PuF, a transcription-promoting factor of the *c-myc* gene [75].

Protein–protein and other Nm23 interaction studies have been complicated by the 'sticky' nature of the molecule, making it difficult to establish specificity [72]. Yet, building upon previous experiments in which histidine kinase activity of NM23 was correlated with reduced metastasis [76], Hartsough et al., showed that Nm23 immunoprecipitated kinase suppressor of Ras (KSR) [77]. KSR is a scaffold protein for the mitogen activated protein kinase (MAPK) cascade. Nm23 phosphorylated KSR at serine 392, a 14-3-3-binding site. This, coupled with observations that Nm23 transfected MDA-MB-435 cells had lower levels of phosphorylated MAPK led to the conclusion that Nm23 signals through the ERK-MAPK pathway [78,79]. Numerous papers have documented signaling through the Ras-ERK-MAPK as important in metastasis. Therefore the KSR result is especially intriguing.

Another interesting interaction involving Nm23-H1 was recently described by Fan et al. [80]. They provide evidence that Nm23-H1 interacts with granzyme A in the process of DNA damage induction in cytotoxic T-cell apoptosis. The mechanism has not been demonstrated in tumor cells; however, the association relates to the NDPK activity of Nm23s and may offer an alternative mechanism for metastasis suppression.

Clinical studies assessing Nm23 as a marker for metastasis were recently reviewed [72]. Briefly, decreased expression (as would be expected for a metastasis suppressor) correlated in many, but not in all cancers. Higher expression in neuroblastoma correlated with aggressiveness. A few studies found no correlation with metastasis. Interpretation is sometimes complicated because each study used different antibodies and involved different criteria. Thus, Nm23 has shown promise for some cancer types, but is not yet considered an independent prognostic factor.

5. KAI-1 (CD82)

KAI-1 was identified in prostate cancer cell lines (Dunning rat AT3.1 and AT6.1) that were suppressed for metastasis following introduction of human chromosome 11 [81]. Positional cloning mapped KAI1 to 11p11.2 [82].

KAI-1 is an evolutionarily conserved member of the tetraspanin transmembrane protein family of leukocyte surface glycoproteins. It is the only tetraspanin with an internalization sequence at the C-terminus [83]. Although no allelic losses were seen, expression in the epithelial compartment was consistently down-regulated during prostate cancer progression [84]. Expression also inversely correlated with breast cancer metastasis [85]. Enforced constitutive expression suppressed metastasis of breast cancer [86] and melanoma [87]. Additionally *KAI1*

inhibited key steps in metastasis (i.e. invasion and motility) of colon cancer cells [88].

There are contradicting reports [89,90] regarding interactions between p53 the *KAI1* promoter following identification of a p53-consensus binding sequence. There is evidence of *KAI1* epigenetic regulation by methylation of CpG islands in the promoter [91]. The mechanism of action is enigmatic, in part, because KAI1 functions as an adhesion molecule on leucocytes, but does dramatically influence adhesion in tumor cells. So, other mechanisms have been proposed. KAI1 directly associates with the EGF receptor and suppresses induced lamellipodia and migration signaling [92]. Attenuation of EGF-induced signaling is accomplished by ligand-induced receptor endocytosis. Thus, KAI1 might suppress metastasis by altering the balance between KAI1 and EGFR, which might affect proliferative and migratory signals delivered. KAI1 also associates with the cytoskeleton promoting phosphorylation and association of both the guanine exchange factor Vav and the adaptor protein SLP76 leading to *de novo* actin polymerization [93]. Involvement of Rho GTPases in KAI1 signaling brings to the forefront additional pathways in KAI1 signaling.

Immunohistochemical detection of *KAI1* correlated inversely with metastasis in many different cancers [59]. Down-regulation of KAI1 was also seen in cancer lines of urogenital, gynecological, and pulmonary origin [94].

6. KISS-1, TXNIP and CRSP3

KISS-1 was identified as a melanoma metastasis suppressor using subtractive hybridization to compare chromosome 6 metastasis-suppressed melanoma hybrids with metastatic parental cells [68,95]. Surprisingly, the *KISS-1* gene mapped to the long arm of chromosome 1 [68]. Enforced expression of *KISS-1* suppressed metastasis of melanoma and breast carcinoma [96]. A deletion variant (neo6qdel; neo6-del(q16.3-q23)) of neomycin-tagged human chromosome 6 did not suppress metastasis and did not express *KISS1* [97]. Therefore, it was hypothesized that regulators of *KISS-1* were encoded on chromosome 6.

Ultimately, the mechanism of action of *KISS-1* remains unknown. Research has been stymied by an apparently short protein half-life. However, three groups studying an orphan G-protein coupled receptor (GPR54, hOT7T175, AXOR12) identified a fragment of *KISS-1* as the ligand [98–100]. KISS-1 fragments were named Metastin [100] and Kisspeptins [98]. The functional peptides were amidated [100]. Ligand binding initiates hydrolysis of (PIP2) and Ca^{+2} mobilization and arachidonate release. ERK1/2 and $p38^{MAPK}$ phosphorylation have also been observed concomitant with cytoskeletal changes [98–102]. Boyd and colleagues showed that constitutive up-regulation of *KISS-1* in HT10810 cells resulted in decreased NFκB activation which, in turn, led to diminution of MMP-9 transcription [103].

While Ohtaki and colleagues showed elegant data showing that exogenous Metastin/Kisspeptin treatment of receptor-transfected B16–BL6 melanoma reduced metastasis and anchorage-independent growth [100], activity of the endogenous receptor has not been demonstrated to date in cancer cells. Likewise, endogenous receptor expression and mutation analysis still need to be done to firmly establish a connection with melanoma metastasis.

The normal physiological function(s) of *KISS-1* (and its receptor) are only recently becoming elucidated. *KISS-1* levels are higher in early placenta and molar pregnancies and are reduced in choriocarcinoma cells, favoring a predominant role in the control of the invasive and migratory properties of trophoblast cells [104].

A clinical role for KISS-1 was inferred by the experimental studies showing metastasis suppression. The following issues have made it difficult to complete a detailed study-lack of antibodies/antisera recognizing KISS-1 or Metastin/Kisspeptin; lack of reagents recognizing receptor; and short life span of the nascent protein. Nonetheless, Shirasaki and colleagues used in situ hybridization to examine *KISS-1* expression in clinical melanoma samples [105]. As expected, an inverse correlation of *KISS-1* with malignancy were found. While carefully performed, information regarding KISS-1 processing or the receptors was not possible in those studies. Importantly, the studies compared LOH on 6q loci with *KISS-1* expression [105]. The clinical studies corroborated the experimental MMCT data linking loci between 6q16.3-q23. Murine orthologs of metastin and GPR54 were used to demonstrate activation of phospholipase C following ligand binding [102].

Recently, Goldberg et al., identified two molecules (TXNIP and CRSP3) that appear to function upstream of *KISS-1* [53]. Briefly, paired microarrays compared metastatic C8161 and non-metastatic neo6/C8161 cells. Also, metastatic neo6qdel/C8161 cells were compared to neo6/C8161. The gene with greatest differential expression in both arrays was VDUP1 (Vitamin D3 upregulated protein 1). VDUP1 was first identified in HeLa cells by differential display following treatment with 1,25-dihydroxyvitamin-D3 [106]. Subsequently it was identified as an interactor of thioredoxin (TRN) in a yeast two-hybrid screen and is also known as TBP2 (TRN binding protein 2) and TXNIP (TRN-interacting protein, preferred name). TRN is a redox- signal regulating protein [107] and regulates stress-response MAPK signaling via suppression of the apoptosis signal-regulating kinase 1 (ASK1) activation and also activation of transcription factors. TXNIP binds to the reduced form of TRN to inhibit function and expression [108,109]. TXNIP also regulates stress-response apoptosis signal transduction [110,111]. Concomitant with increased *TXNIP* expression is decreased expression of TRN and arrest of cell growth [112]. Based upon trends toward increased TRN in many tumors and cell lines, TXNIP may have tumor suppressor effects as well.

CRSP3 encodes a co-factor required for SP1-mediated activation of transcription. Sp1 (Specificity protein 1) is a general transcription factor that binds to and acts through GC-boxes, widely distributed promoter elements [113,114]. *CRSP3* has no known yeast or murine orthologs [115]. Definitive clinical studies have not yet been done, but *CRSP3* and *TXNIP* expression, generally inversely correlate with melanoma progression. Additionally, sequence tagged sites adjacent to *CRSP3* in patient samples [105] suggest that the gene may indeed show changes associated with clinical outcome.

7. TIMPs

Tissue inhibitors of metalloproteinases (TIMPs) are a family of secreted proteins that selectively, but reversibly, inhibit metalloproteinases (MMPs) with 1:1 stoichiometry [10,116,117]. Modulation of MMP and TIMP levels is critical to the control of extravasation and tumor-induced angiogenesis, processes that involve basement membrane degradation. Paradoxically, TIMP-1, 2 and 4 have an anti-apoptotic effect, while TIMP-3 induces apoptosis. TIMP-2, in concert with MT1-MMP can bind to and activate proMMP-2 (reviewed in Ref. [116]). Although there are no known TIMP-specific receptors, membrane-bound molecules such as MT-MMPs and metalloproteinase disintegrins (ADAMs) serve as TIMP-binding molecules at the cell surface [117].

TIMPs are expressed in tumor tissues and are present in the sera of cancer patients, raising the possibility that TIMP levels could predict clinical outcome and risk of metastasis [118–121]. But results are complicated because the ratio of TIMPs to MMPs is the crucial parameter. Nonetheless, the possibility that serum TIMP levels could be useful in a clinical setting remains. Gene therapy studies for local or systemic delivery of TIMPs are in an exploratory phase (reviewed in Ref. [122]).

8. Cadherins

Cadherins are transmembrane glycoproteins responsible for Ca^{+2}-dependent cell adhesion. Although the family is widely expressed, E-cadherin (gene designation CAD1) is expressed on epithelial cells. A precursor protein (135 kDa) is processed to a mature 120 kDa form. The extracellular N-terminus is critical for homophilic Ca^{+2}-dependent cell–cell adhesion. The C-terminus interacts with β-catenin to mediate actin binding. E-cadherin/β-catenin binding sequesters the latter, blocking nuclear translocation and transcription of *c-myc* and *cyclin D1*.

Defining a role for E-cadherin as a metastasis suppressor is complicated. Over-expression decreases motility and invasiveness [123]. Mutations of CAD1 and α-catenin have been associated with invasion [124]. High E-cadherin levels inhibit shedding of tumor cells from the primary tumor; thus, CAD1 is a metastasis-suppressor [124–126]. However, CAD1 can also be a tumor suppressor [124,125,127]. Loss of expression occurs in many tumors and is directly associated with loss of differentiation (reviewed in Ref. [128]). Mechanisms of reduced expression include: reduction or loss of E-cadherin expression (by LOH or epigenetic silencing [129]), redistribution to different sites within the cell, shedding of E-cadherin

and competition from other proteins (reviewed in [130]). Stimulation of the EGFR by EGF, TGF-β or PP2 brings about phosphorylation of E-cadherin and β-catenin resulting in dissociation of the complex [131,132]. Other than breast and gastric cancers, with nearly 50% of the tumors affected, mutations of *CAD1* appear to be infrequent [133]. Evidence supports a role of E-cadherin in tumor suppression rather than just being an epiphenomenon of the tumor cells' phenotypic changes [134]. Since loss of E-cadherin alone, leading to decreased cell–cell adhesion is insufficient for the tumor cells to invade, it appears more than likely that down-regulation actively transduces specific signals that support tumor invasion.

Recently, Kashima et al., showed that N-cadherin and cadherin-11 (osteoblast cadherin), which are both highly expressed in osteoblasts (bone forming cells), reduce metastasis to lungs without negatively affecting tumorigenicity [135]. Reduced motility was presumably the mechanism responsible for diminished metastasis. Curiously, N-cadherin and cadherin-11 are frequently over-expressed in many metastatic breast and prostatic carcinoma cells [136–138]. Moreover, transfection and over-expression promotes invasion and metastasis in breast and melanoma cells [136,139,140]. These results highlight the complexities of interpretation because of cell origin. They further reinforce the point raised above—gene context is important.

9. MKK4

MKK4/JNKK1/SEK1 is a mitogen-activated protein kinase, which transduces signals from MEKK1 to stress-activated protein kinase/JNK1 and $p38^{MAPK}$ [59]. MKK4 transmits stress signals to nuclear transcription factors that mediate proliferation, apoptosis and differentiation. Portions of the *MKK4* gene (on chromosome 17) were deleted or altered in cancer cell lines that displayed defects in signal transduction from MEKK1 [141]. Suppression of prostate cancer cell metastasis was brought about by over-expressed *MKK4* [142]. An inverse relationship between Gleason score and MKK4 staining was established in prostate tumors [143]. *MKK4* is also a metastasis suppressor in ovarian carcinomas [144].

10. BRMS1

Following upon MMCT studies, Seraj et al., performed differential display to identify the gene(s) responsible for chromosome 11 suppression of breast cancer metastasis. Three novel cDNAs were identified. *BRMS1* suppressed metastasis in MDA-MB-231 and MDA-MB-435 [145] breast carcinomas in addition to two human melanoma (C8161 and MelJuSo, [146]) and two murine mammary carcinoma cell lines (4T1 and 66cl4 [147]). *BRMS1* transfectants were not suppressed for growth in vitro or in vivo; adhesion to extracellular components (LN, FN, collagens I or IV, Matrigel); expression of gelatinases (MMP-2, MMP-9) or heparanase, or invasion in vitro [148].

The *BRMS1* gene mapped to human chromosome 11q13.1-q13.2, a region frequently altered in metastatic breast cancer. Expression of other metastasis suppressors (i.e. *NM23*, *KAI-1*, *KISS-1*, *CAD1*) did not correlate with *BRMS1*. Motility was moderately reduced in wound assays as was the ability to grow in soft agar. The most striking change amongst transfectants was restoration of gap junctional intercellular communications (GJIC) [148,149], accompanied by increased expression of connexin (Cx) 43 and decreased expression of Cx32 [150]. Connexins are the protein subunits of gap junctions and the expression pattern in *BRMS1* transfectants more closely mimics normal breast tissue. Using real time RT-PCR, *BRMS1* expression inversely correlated with metastasis in human melanoma cells [146]. Expression of *BRMS1* also reduced metastasis of T24T, a metastatic variant of the human bladder carcinoma cell line, T24 [151]. Although a role in normal physiology has not been determined, *BRMS1* does not appear to regulate invasive and/or migratory properties of trophoblast cells [104]. BRMS1 RNA expression was detected in villous cytotrophoblasts, but the level in invasive cytotrophoblasts, the subclass of trophoblast cells that invades into the decidua was not examined, thus warranting prudence in interpreting the data.

Hunter and colleagues [152,153] using a genetic approach to identify factors predisposing to metastatic disease, co-localized the *Brms1* gene with the *Mtes1* (*Metastasis Efficiency Suppressor 1*) locus on chromosome 19 (orthologous to human chromosome 11). Later studies utilizing comparative sequence

analysis, however, suggest that *Brms1* is not likely *Mtes1* [152,154].

11. SSeCKS

SSeCKS (pronounced essex) for Src-suppressed C kinase substrate expression is down-regulated in *src*- and *ras*-transformed rodent fibroblasts [155,156]. It is the likely rodent ortholog of human Gravin/AKAP12, a cytoplasmic scaffolding protein for protein kinases A and C [157], concentrating at the cell edge and podosomes. In response to phorbol esters, *SSeCKS* controls elaboration of a cortical cytoskeletal matrix. Over-expression suppresses v-src-induced morphological transformation and tumorigenesis. ERK2 activity was induced 5- to 10-fold in presence of v-src [158], resulting in decreased cyclin D1 expression and pRb phosphorylation, thereby playing a role cell cycle progression [158,159]. While SSeCKS/Gravin protein is detected in untransformed rat and human prostate epithelial cell lines, expression is severely reduced in metastatic prostate carcinoma cell lines. Re-expression significantly decreased lung metastases, induced filopodia-like projections and decreased anchorage-independent growth [160] in vitro.

12. RhoGDI2

Rho GTPases are guanine nucleotide binding proteins, which cycle between active GTP-bound state and inactive GDP-bound state. RhoGDI (Rho *GDP dissociation inhibitors*) stabilize the GDP-bound form and sequester them in an inactive non-membrane localized, cytoplasmic compartment [161]. In an earlier bladder carcinoma study, RNA expression of RhoGDI2 was associated with decreased metastatic potential [151]. Transfection and enforced expression suppressed metastasis of T24 human bladder carcinoma variants [162]. Gene expression profiling of 105 bladder carcinomas, corroborated the expression pattern—i.e. *RhoGDI2* expression correlated inversely with the invasive phenotype of tumors.

13. Drg-1

Drg-1 (a.k.a. RTP, cap43 and rit42) was identified as a differentiation-associated gene in colon carcinomas by differential display [163]. It is orthologous to mouse TDD45 and Ndr1 and rat Bdm1. Kurdistani and colleagues showed that introduction of *Drg-1* cDNA suppressed tumorigenicity of human bladder carcinoma cells, suggesting that *Drg-1* is a tumor suppressor gene [164]. However, in vitro invasion and liver metastases are inhibited from colorectal carcinomas when expression is restored either by inhibiting DNA methylation or by transfection [165]. Likewise, Bandopadhyay et al., recently showed that prostate carcinoma cells are suppressed for metastasis, but not tumorigenicity, when Drg-1 is over-expressed [166]. The latter studies support the contention that *Drg-1* is a metastasis suppressor.

Drg-1 expression inversely correlated with Gleason score in human prostate cancer specimens [166]. While the mechanism of action of *Drg-1* is unknown, it is up-regulated by PTEN and p53 and phosphorylated by Protein Kinase A [167]. It is postulated that Drg-1 might function downstream of MKK4, since it is induced similarly to the stress activated protein kinases (JNK/SAPK) [168] via MKK4, itself a metastasis-suppressor.

14. Metastasis suppressors without functional portfolio

The above genes have functional evidence supporting classification as metastasis suppressors. We will briefly describe below several others whose evidence is suggestive, but the data are deficient with regard to classification as metastasis suppressors for two reasons. First, the data are at this time correlative, not functional. Second, functional suppression of metastasis occurs concurrent with diminished tumorigenicity. In the absence of experimental arms to accommodate differential growth rates and detailed analysis to verify expression, designation as metastasis suppressors by the strict definition is not possible.

Responding to environmental and growth stimuli, axons extend growth cones in several directions. *Semaphorins*, a large family of secreted

and membrane-bound proteins participate in a repulsive (collapse) process [169,170]. CRMP proteins aid intracellular transduction of collapse signals [171]. *CRMP-1*, for *Collapsin Response Mediator Protein-1*, is one of five proteins in the CRMP family, whose molecular mechanisms have not yet been characterized, although recent literature implicates involvement in controlling cell movement (reviewed in Ref. [172]). Recently, CRMP-1 was shown to reduce invasion of lung cancer cells [51]. Shih et al., demonstrated that CRMP-1 expression was inverse to lung carcinoma grade. Expression correlated directly with survival and time to relapse.

Gelsolin modulates actin assembly and disassembly to regulate motility. It also inhibits apoptosis [173]. Gelsolin decreases colonization in soft agar, retards spread, reduces chemotaxis to fibronectin and suppresses both tumorigenicity and metastasis of melanoma [174], bladder carcinoma [175] and lung carcinoma [176].

Following identification by DD-RT-PCR comparing normal mammary epithelium and invasive mammary carcinoma cells, *maspin* (mammary serine protease inhibitor) was reported to suppress invasion and metastasis (but no metastasis data was shown in the original paper). Complicating interpretation, tumorigenicity and growth were also reduced. [177]. The gene, *SERPINB5*, is a member of the serine protease inhibitor (serpin) gene cluster on chromosome 18q21.3. Maspin transgenic mice show attenuated tumor progression and metastasis, supporting its role against tumor spread [178]. Mechanistically, maspin also sensitizes cells to induced apoptosis [179] and reduces angiogenesis [180]. Expression of maspin is controlled at several levels. Futscher et al. [181] showed that cell-type specific expression of maspin inversely correlated with methylation of *SERPINB5*. *SERPINB5* expression can be surmounted by treatment with 5-aza-2′-deoxycytidine [182]. Regulation of maspin by p53 has also been reported using EMSA [183].

Heterochromatin-associated protein 1 ($HP1^{HS\alpha}$) expression is down-regulated in highly invasive metastatic cells compared to non-metastatic cells where it is predominantly localized in the nucleus. Although the clinical correlations show promise as a metastasis suppressor HP1 in breast carcinoma [184], no data functional evidence for metastasis suppression are yet available.

Data for *CD44* as a metastasis suppressor are controversial. Gao et al., showed CD44 to have metastasis suppressor activity in AT3.1 prostate carcinoma cells, without altering tumorigenicity [185]. Complexity exists because CD44, which encodes a membrane protein that binds the extracellular membrane components hyaluronic acid and osteopontin exists in multiple isoforms. The standard isoform, CD44-s, significantly (>60%) reduces lung metastases, but it is still not certain which are the most relevant isoforms for cancer and metastasis. Reagents to study the role(s) of particular isoforms in tumorigenicity and/or metastasis are under development. Until then, CD44 data should be interpreted cautiously.

SHP-2 is a widely expressed cytoplasmic tyrosine phosphatase that is believed to participate in signal relay downstream of growth factor receptors. SHP-2 impairs spreading of fibroblasts on fibronectin and migration (in vitro) [186]. Cells expressing mutant SHP-2 display reduced focal adhesion kinase dephosphorylation as well as decreased association with paxillin. In vivo demonstration of metastasis suppression remains to be done.

15. Remaining questions and perspectives

The critical clinical threshold for any cancer is development of metastasis. Diagnosis occurring prior to the establishment of secondary lesions means favorable prognosis and more effective treatment. As a result, earlier, more effective diagnosis has been instrumental in improving cure rates for cancer.

Unfortunately, there are many cases in which there is no evidence of cancer spread at the time of diagnosis. Treatment plans are usually based upon somewhat subjective morphologic criteria in tissue specimens submitted to the pathologist. In the case of breast cancer, approximately 25% of node-negative patients develop metastases despite being designated 'metastasis negative' at the time of diagnosis. What can be done to identify the patients whose cancers are likely to spread and those whose cancers are unlikely to form secondary lesions? The answer depends upon

a thorough understanding of the underlying genetic and biochemical basis of metastasis.

While it is not yet known how, or whether, metastasis suppressor genes will play a role in predicting the propensity to metastasize in clinical cancer, information gained by understanding the mechanisms of action of the metastasis suppressors is providing insight into the fundamental mechanisms controlling cancer spread. The metastasis suppressors identified in Table 1 and Fig. 1 were discovered in several laboratories, using different model systems, and tested using distinct experimental systems. There is variability in terms of understanding mechanism and with regard to clinical evaluation. Nonetheless, the pieces to a complex jigsaw puzzle are beginning to take form. Pathways are beginning to emerge that connect heretofore independent metastasis suppressors. The picture is still sketchy; but some common elements are apparent.

First, many metastasis suppressors have functions that amplify 'signals' (i.e. there are several branches downstream in each signaling arbor). This situation is highly desirable for controlling complex, multigenic phenotypes like metastasis. Second, metastasis suppressors exist within all cellular compartments. The situation is reminiscent of the genes controlling cell cycle, apoptosis, and differentiation. The expectation (hope?) is that, like the cell cycle genes, some higher order will become evident as the regulatory molecules are put into pathways. Moreover, it is hoped that key rate-limiting steps will be identified. Third, many metastasis suppressors function in diverse cell types (i.e. genes discovered in one tumor type also suppress metastasis in cells of other origins). Fourth, despite use of a strict definition of metastasis suppression (i.e. demonstration of a functional suppression of metastasis without inhibition of tumor formation), the number of metastasis suppressor genes is continuing to grow. How many metastasis suppressor genes are there? We do not know. Based upon similarly highly regulated phenotypes, we would predict that the number is limited within the core regulatory pathway(s). The complexity is daunting if alterations downstream are also counted.

Table 1
Characteristics of metastasis suppressor genes

Gene	Method of discovery[a]	In vitro characterization[b]				In vivo characterization		
		Soft agar colonization	Motility	Invasion	Adhesion to ECM components	Tumor growth	Metastasis	Clinical specimens
BRMS1	MMCT/DD	↔	↓	↓	↓	↔	↓	
CAD1	Clin		↓		↓	↔	↓↑	↓
Cadherin-11	MA		↓			↔	↓↑	
CD44-s	MMCT					↔	↓	↓
CRMP-1	MA			↓		↔		↓
CRSP3	MMCT/MA					↔	↓	
Drg-1	DD	↔	↓	↓	↓	↔	↓/↔	↓
Gelsolin	Clin	↓				↓	↓	
$HP1^{HS}\alpha$	Clin			↓				↓
KAI-1	SH	↓	↓	↓	↔	↓	↓	↓
KISS-1	MMCT/SH	↓	↓	↓	↔	↔	↓	↓
MKK4	MMCT/PC					↔	↓	
N-cadherin	MA					↔	↓↑	
NM23	SH	↓	↓	↓	↓	↔	↓	↓↑
RhoGDI2	MA			↓		↔	↓	↓
SERPINB5	DD		↓	↓		↓	↓	
TXNIP	MMCT/MA				↔	↔	↓	↓

[a] The method of discovery is abbreviated: Clin, clinical correlation; DD, differential display; MA, microarray; MMCT/DD, microcell-mediated chromosome transfer + differential display; or SH, subtractive hybridization.

[b] Arrows depict direction of change in behavior or expression (in clinical samples). (↔) depicts no consistent change. Fields left blank indicate that the experiments have not yet been done or have not been reported.

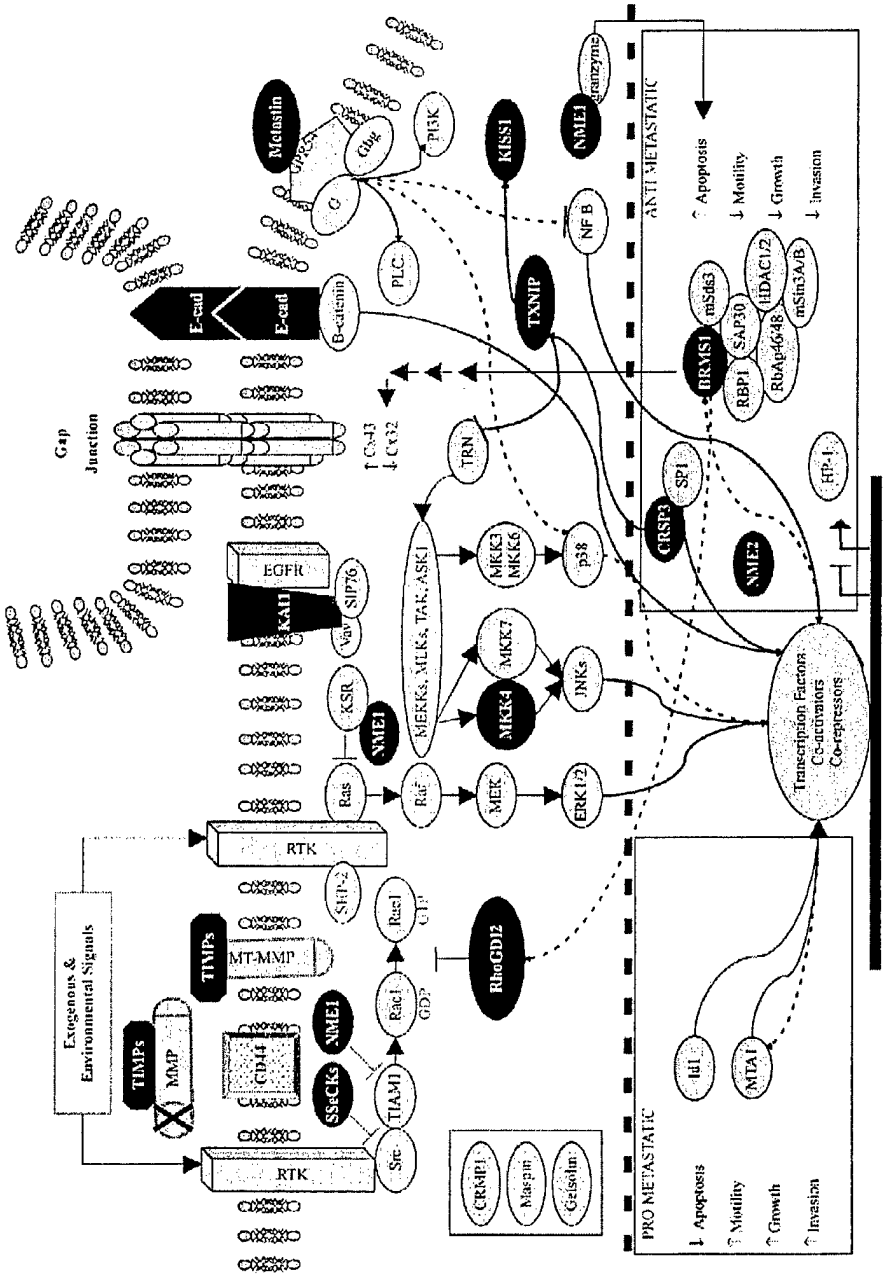

Fig. 1. Proposed model of pathways of metastasis suppression. Metastasis suppressor genes are featured in solid black shapes. Solid lines indicate pathways for which biochemical evidence has been provided. The dotted lines represent inferred/implied pathways. Putative metastasis suppressors are stippled. If the location of the proteins are known, they are placed. If the location or functional subcellular compartment are not definitively known, the proteins are placed in the box at the left. Some connections are omitted to simplify the figure. The shadowed boxes positioned within the nucleus highlight the existence of pro- and anti-metastatic genes involved in transcription. Two molecules are highlighted with regard to promoting metastasis, Id1 [199,200] and MTA1 [201,202].

The field of metastasis genetics and the existence of genes that specifically control metastasis has been called into question by some [6,7]. Yet, functional data with the metastasis suppressor genes strongly argue that there are specific genes controlling metastasis.

Our colleague, Kent Hunter has collected some very important data that support the existence of metastasis genes using breeding strategies in mice. Using a transgene-induced mouse mammary tumor model (MMTV-PyMT), mice were crossed with mice of varying genetic backgrounds. Significant differences in metastasis were found despite failure to alter tumor initiation or growth kinetics in some strains. Since all of the mouse tumors were initiated by the same oncogenic event, the differences in metastasis and gene expression are most likely due to genetic background. His data reinforce a notion that we introduced earlier—gene context is an important parameter in determining metastatic potential.

Further contributing to the argument that microenvironment is important are observations from multiple laboratories showing that many metastasis suppressors act at the terminal steps of the metastatic cascade, i.e. proliferation at the secondary site [34,67, 187]. In studies from our laboratory, we have showed, that tumor cells proliferated in some sites (i.e. orthotopic) but not others (i.e. metastatic). Furthermore, we have preliminary evidence that some metastasis suppressor genes suppress colonization in some organs, but not others (J.F. Harms and D.R. Welch unpublished). Much more work will be required to understand the interplay between metastasis-controlling genes and microenvironment; however, the importance of cellular context cannot be overstated.

An issue that has stymied the field for several years is the imprecise use of terminology. Even a cursory look at the literature finds numerous papers that claim suppression of metastasis. Many claims are unfounded because there is no biological data to support them. Metastasis is an in vivo phenotype and, quite simply, in vitro assays are not always predictive of in vivo behavior. In short, many labs suppressed steps of metastasis (i.e. invasion, motility, adhesion, resistance to apoptosis, growth) without testing the impact of changes using in vivo metastasis assays. Correlative studies are often related to promises unfulfilled. Nonetheless, we are encouraged by the emergence of new researchers in the metastasis field and the breadth of expertise that they bring. More common are claims that a gene blocks metastasis when it blocks growth—tumorigenicity. The issue was addressed above. However, the field must address the paradox that emerges when metastasis is suppressed in one cell type but tumorigenicity is suppressed in another (as for E-cadherin and DRG-1).

What do the data summarized in this review tell us about the clinical control of metastasis? Readers are cautioned to note that reliable antibodies/antisera recognizing many of the metastasis suppressors do not yet exist. As a result, many of the correlations presented are measured using RNA. While proportional expression of RNA and protein is anticipated for most, data are not yet available to definitively conclude such. Likewise, it is not known whether some metastasis suppressors are post-translationally modified. Ultimately, interpretation will depend upon identifying the functional protein responsible for metastasis suppression.

Another area of active research relates to the mechanisms responsible for loss of metastasis suppressor gene expression. Both anecdotal and published data suggest that many metastasis suppressor genes are not mutated, but are differentially expressed (reviewed in Ref. [188]). While not described in detail here, there are several levels at which expression could be regulated—protein translation [189,190], methylation [191,192], histone acetylation [192–195], mRNA protein stability [196,197]. Pat Steeg and colleagues have been pioneering the notion that metastasis suppressor genes may be re-expressed in a clinical setting. Recent data from her laboratory show that dexamethasone and medroxyprogesterone acetate can enhance expression of Nm23 [198]. They have also presented evidence that hypomethylation by 5-azacytidine can restore Nm23 expression as well [79]. While data were not collected for the other metastasis suppressors, their data support the possibility of pharmacologic regulation of metastasis via metastasis suppressor genes. Given that the drugs used for their experiments are first line, the possibility for therapeutic intervention in the near term is very real.

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Breast Cancer Cells Induce Osteoblast Apoptosis: A Possible Contributor to Bone Degradation

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Abstract Breast cancer cells exhibit a predilection for metastasis to bone. There, the metastases usually bring about bone loss with accompanying pain and loss of function. One way that breast cancer cells disrupt the normal pattern of bone remodeling is by activating osteoclasts, the bone degrading cells. Nevertheless, targeting the osteoclasts does not cure the disease or result in bone repair. These observations indicate that osteoblast function also may be compromised. The objective of this study was to investigate the interaction of metastatic breast cancer cells with osteoblasts. Human metastatic breast cancer cells, MDA-MB-435 or MDA-MB-231, or their conditioned media were co-cultured with a human osteoblast line hFOB1.19. The breast cancer cells caused an increase in the prevalence of apoptotic osteoblasts. Apoptotic osteoblasts detected by the TUNEL assay or by caspase activity increased approximately two to fivefold. This increase was not seen with non-metastatic MDA-MB-468 cells. In an investigation of the mechanism, it was determined that the hFOB1.19 cells expressed fas and that fas was functional. Likewise the hFOB1.19 cells were susceptible to TNF-α, but this cytokine was not detected in the conditioned medium of the breast cancer cells. This study indicates that osteoblasts are the target of breast cancer cell-induced apoptosis, but fas/fas-ligand and TNF-α, two common initiators of cell death, are probably not involved in this aspect of the metastases/bone cell axis. There are several mechanisms that remain to be explored in order to determine how breast cancer cells bring about osteoblast apoptosis. Even though the specific initiator of apoptosis remains to be identified, the results of this study suggest that the mechanism is likely to be novel. J. Cell. Biochem. 91: 265–276, 2004.

Key words: apoptosis; osteoblasts; breast cancer; metastasis; bone

Bone is the most common destination of breast cancer metastases. In nearly 50% of first metastasis and in greater than 70% of re-occurrences, breast cancer cells colonize the bone [Rubens and Mundy, 2000]. The result is extensive bone degradation with accompanying pain and loss of function. While the breast cancer cells themselves may degrade bone, especially late in metastasis [Sanchez-Sweatman et al., 1998] it is now widely accepted that the breast cancer cells also upset the normal bone remodeling process such that the osteoclasts become hyperactive. Guise and co-workers [Guise, 2000] present a model in which breast cancer cell-derived parathyroid hormone related protein (PTHrP) is indirectly responsible for the activation of osteoclasts. PHTrP activates osteoblasts to produce a receptor activator of nuclear factor κB ligand (RANKL) which, in turn, activates osteoclasts to degrade bone,

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thereby releasing TGF-β from the matrix. TFG-β in turn stimulates the cancer cells to produce more PTHrP setting up a "vicious cycle." More recently, other models have indicated that IL-8 [Bendre et al., 2002] and endothelin are critical molecules [Guise et al., 2003]. There are numerous other molecules in the metastatic environment that undoubtedly play a role [Mundy et al., 2002].

Because of their direct role in bone degradation, osteoclasts are the major target of pharmaceutical interventions. Several derivative drugs of the bisphosphonate family have succeeded in slowing lesion progression, but they do not bring about a cure. Moreover, the already existing lesions do not heal [Lipton et al., 2000]. Sasaki et al. [1995], using a rodent model and lytic MDA-MB-231 human breast cancer cells, carried out a histomophometric analysis following a bisphosphonate (residronate) treatment. Despite the fact that residronate reduced osteoclast numbers, slowed bone lysis, and reduced the tumor burden, there was no evidence for bone deposition and repair. This outcome suggests that normal osteoblast function also is impaired in osteolytic metastasis. In fact, very little is known about the osteoblast in osteolytic breast cancer metastasis. Furthermore, the clinical model is probably more complex than the mouse model. There are few studies of osteoblasts during bone metastasis. Stewart et al. [1982] carried out quantitative, histomorphometric analysis of bone biopsies of patients with hypercalcemia due to metastasis. Although there was the expected increase in osteoclast numbers overall, there was also a significant decrease in osteoblasts, osteoid surface, absolute osteoid volume, and an increase in empty osteocyte lacunae in bone adjacent to the tumor. Others report diminished or abnormal osteoblast activity near the site of the metastases [Taube et al., 1994]. These data taken together indicate a decrease in normal osteoblast function.

These reports led us to question the impact of breast cancer cells on osteoblasts. We developed a model co-culture system using an immortalized human fetal osteoblast cell line, hFOB1.19, and human metastatic breast cancer cells, MDA-MB-231 and MDA-MB-435 [Mercer et al., 2003]. Early in the course of these studies we observed an increase in osteoblast apoptosis in the presence of breast cancer cells. The results of the study to verify this observation are reported here.

MATERIALS AND METHODS

Cell Culture

The human fetal osteoblast line, hFOB1.19, immortalized with SV40 large T antigen [Harris et al., 1995], was a generous gift from Dr. Thomas Spelsberg. The cells were maintained in a 34°C, 5% CO_2, humidified chamber, with growth medium consisting of DMEM:Ham's F-12 (1:1), 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin, hereafter referred to as "growth medium." To obtain differentiated osteoblasts, 85–90% confluent cultures were changed to differentiation medium and transferred to a 39.5°C, 5% CO_2, humidified chamber for 2 or 3 days before treatment as indicated in individual experiments. Differentiation medium consisted of DMEM:Ham's F-12 (1:1), 10% charcoal-stripped FBS, 50 μg/ml vitamin C (ascorbic acid), 10^{-8} M vitamin D_3, and 10^{-8} M vitamin K_3 (menadione).

Two human breast cancer metastatic cell lines, MDA-MB-231 and MDA-MB-435, and one non-metastatic breast cancer cell line, MDA-MB-468 [Price, 1996] were used. They were stably transfected with the plasmid pEGFP-N1 [Harms et al., 2002]. They could easily be distinguished from the hFOB cells in co-culture by fluorescent microscopy. MDA-MB-231 and MDA-MB-435 cells were maintained in DMEM supplemented with 5% FBS in a humidified 37°C chamber with 5% CO_2. Stock culture medium was supplemented with 500 μg/ml G418 to maintain pEGFP-N1. MDA-MB-468 cell cultures were maintained in DMEM:-Ham's F-12 supplemented with 10% FBS and 400 μg/ml hygromycin.

WR19L-A12, a mouse lymphoma cell line, stably transfected with human fas-ligand [Tanaka et al., 1998] was a generous gift from Dr. Laurie Owen-Schaub. WR19L-A12 cells were maintained in RPMI 1640 medium with 4 mM L-glutamine and 23.81 mM sodium bicarbonate, 10% FBS, 1 mM sodium pyruvate, 1% penicillin and streptomycin in a humidified 37°C chamber with 5% CO_2. Stock cultures were supplemented with 400 μg/ml G418 to select for fas-ligand expressing cells.

Breast cancer cell conditioned medium was prepared from breast cancer cells plated in T 75 cm^2 tissue culture flasks with 10 ml DMEM

supplemented with 5% FBS and incubated at 37°C. When the cells were nearly confluent, the medium was changed to serum-free DMEM:-Ham's F-12 (1:1). After 24 h the media were collected, aliquoted and frozen at −20°C. For most experiments they were diluted 1:1 with differentiation medium. In some experiments, as indicated, DMEM:Ham's F-12 was supplemented with 5% Serum Replacement 1 (Sigma, St. Louis, MO) and allowed to remain with the breast cancer cells for 3 days. Vehicle conditioned medium was prepared the same way as conditioned media except that it was not exposed to cells. Control conditioned medium was collected from NIH 3T3 fibroblasts cultured in the same way as for the breast cancer cells.

Apoptotic Analysis

Apoptosis was detected by one of two methods depending on the experiment. Caspase-3/7 activity was assayed with the APO-One™ Homogeneous Caspase-3/7 Assay (Promega, Madison, WI). Fragmented DNA was measured by a modified TUNEL assay [Jewell and Mastro, 2002]. Briefly, terminal deoxynucleotidyl transferase (TdT) from Promega was fluorescently linked to FluoroLink™ Cy5-dUTP (Amersham Pharmacia, Piscataway, NJ) for microscopy or to Biotin-21-dUTP (Clontech Laboratories, Inc., Palo Alto, CA) and R-phycoerythrin(RPE)-Cy5-conjugated streptavidin (DAKO, Copenhagen, Denmark) for flow cytometry. SYTOX® Orange Nucleic Acid Stain (Molecular Probes, Eugene, OR) was used to visualize the nuclei of all cells by fluorescence microscopy.

Caspase Activity

In order to assay caspase activity, hFOB1.19 cells were plated in T 75 cm^2 tissue culture flasks at 10^6 cells per flask and treated as described for individual experiments. After the final incubation, the cells were rinsed with PBS and harvested with 5 ml of Accutase™ (Innovative Cell Technologies, Inc., San Diego, CA). All media and washes were combined in order to collect all of the cells. Cells were centrifuged (4°C, 300*g*), resuspended in growth medium, and filtered through a 40 μm mesh nylon screen (Small Parts, Inc., Miami Lakes, FL) to ensure a single cell suspension. Cells were counted with a hemacytometer and 5×10^5 cells were placed in the wells of a 96-well plate in 100 μl of medium. Next, 100 μl of homogeneous caspase-3/7 reagent was added to each well. After shaking (30 s, 300 rpm), the plate was incubated (room temperature, dark) and readings (excitation/emission, 499/521 nm) were taken every hour for up to 6 h to obtain relative caspase 3/7 activity.

Flow Cytometric Analysis of TUNEL

Cells were plated and incubated as described for the caspase assay. After the final incubation in T-75 flasks, the cells were released with Accutase™, washed twice in PBS (4°C, 300*g*), fixed in 2% paraformaldehyde (20 min, 4°C), washed three times in PBS, and resuspended in 70% ethanol overnight at −20°C for permeabilization. The suspension was centrifuged to remove ethanol and the cells were washed three times with PBS before continuing with the TUNEL assay. At this point the cells were transferred to 12 by 75 mm plastic test tubes compatible with the CoulterXL flow cytometer in order to reduce cell loss. The protocol for DeadEnd™ Fluorometric TUNEL System (Promega) was followed for non-adherent cells with modifications for the fluorescent nucleotide mix. Biotin-21-dUTP (Clontech) was substituted for fluorescein dUTP in the nucleotide mix. A second incubation with streptavidin-RPE/Cy5 (488/670 nm) (DAKO) was carried out after the enzyme reaction was terminated and cells were washed in 0.1% Triton®X-100 in PBS containing 5 mg/ml bovine serum albumin. Propidium idiode staining was omitted. The cells were washed and resuspended in PBS for analysis.

Microscopic Analysis of TUNEL

In order to perform the TUNEL assay, hFOB1.19 cells were plated on gelatin, 0.5 mg/ml, coated glass coverslips (12 × 0.17 mm) in 24-well plates at 10^4 cells per well and treated as described for individual experiments. The cells were washed three times with PBS and fixed with 2% paraformaldehyde. Coverslips were processed in 24-well plates. Samples were rinsed three times with PBS before permeabilizing the cells with 2% Triton®X-100. The protocol for the DeadEnd™ Fluorometric TUNEL System (Promega) was followed for adherent cells except that the fluorescent nucleotide mix was replaced with the same mix containing Cy5-dUTP instead of fluorescein. After the samples were observed by microscopy and images captured for GFP (488/509 nm) and Cy-5 (649/670 nm), the slide was stained with SYTOX®

Orange in place of propidium iodide as described [Jewell and Mastro, 2002].

To calculate the percentage of apoptotic hFOB1.19 cells, digital images were captured on the Olympus BX-60 widefield digital microscope fitted with fluorescence optics. At least 700 fields per sample were captured for analysis of TUNEL positive cells. The number of breast cancer cells per sample was calculated by observing 10–50 images per slide and multiplying the average number of GFP positive cells per field by the total number of fields analyzed for apoptosis. The percentage of apoptotic cells was calculated from the total numbers of cells on the coverslip.

Materials

Sources of materials not already indicated are provided here. Biotin conjugated mouse anti-human monoclonal antibody CD95 (clone DX2), biotin conjugated mouse anti-human fas-ligand monoclonal antibody (clone NOK-1), biotin-conjugated mouse IgG1,κ immunoglobulin isotype control (clone MOPC-21), and purified mouse anti-human TNF-α monoclonal antibody (no azide, low endotoxin, MABTNF-A5), were purchased from BD Biosciences (San Diego, CA). Vitamin D_3 was purchased from BIOMOL Research Laboratories, Inc., (Plymouth Meeting, PA). Caspase-3 Inhibitor (Ac-DEVD-CHO) and GM-6001, a metalloproteinase inhibitor, were obtained from Chemicon International (Temecula, CA); and human CD95 biotin conjugate (Clone B-G27) and mouse isotype control for flow cytometry (mouse IgGγ2a biotin) were from Cymbus Biotechnology Ltd., (Chandlers Ford, Hants, UK). Hygromycin B and G418 were from Calbiochem (EMD Biosciences, Inc., La Jolla, CA). From Immunotech (Beckman Coulter, Brea, CA), we purchased mAb CD95 fas (clone CH-11). Cell Titer 96® Aqueous Assay was purchased from Promega. Heat inactivated FBS, MTT, menadione (vitamin K_3), and human recombinant tumor necrosis factor-alpha (TNF-α) were purchased from Sigma. Vectashield Mounting Medium was a product of Vector Laboratories (Burlingame, CA).

RESULTS

Detection of Apoptotic Osteoblasts

In order to determine if metastatic breast cancer cells were able to attach and grow on monolayers of osteoblasts, GFP expressing MDA-435 or MDA-231 cells were added to confluent monolayers of hFOB1.19 cells. Before viewing with a fluorescence microscope, the cultures were stained with propidium iodide as a means of identifying dead cells. The green fluorescent breast cancer cells could clearly be seen growing over the hFOB cells (Fig. 1A) which were evident when a phase contrast objective was used. The MDA-435 cells were elongated and followed the underlying osteoblasts; the MDA-231 cells remained more compact (Fig. 1B). Although most osteoblasts had a typical morphology, the propidium iodide stain revealed the presence of apoptotic nuclei in some osteoblasts (Fig. 1 arrows). Apoptotic nuclei also were apparent when the cultures were stained with DAPI or Hoechst stain (micrographs not shown).

Quantification of Apoptotic Osteoblasts

In order to quantify the percentage of apoptotic nuclei and to ascertain that they belonged to hFOB cells and not breast cancer cells, we carried out TUNEL assays of the culture using Cy5-dUTP to distinguish the fluorescence of the apoptotic nuclei from that of the GFP. In addition Sytox® Orange was used to stain all the cells in the culture in order to calculate the percentages of apoptotic osteoblasts and breast cancer cells. The monolayers of hFOBs cultured alone contained about 2% apoptotic cells. Breast cancer cell cultures were similar, between 1 and 2%. In co-culture the percentage of apoptotic hFOB cells increased several fold (8–12%) while the percentage of apoptotic breast cancer cells did not change (Fig. 2). Similar results were obtained with MDA-MB-231 cells. However, co-culture with non-metastatic MD-MBA-468 cells did not bring about an increase in apoptotic hFOB cells.

Quantitation of TUNEL positive hFOB1.19 cells after incubation with conditioned medium was also carried out by flow cytometry (Table I). Compared to vehicle conditioned medium, i.e., medium prepared like cell conditioned medium, but without cells, apoptosis increased about sixfold.

Caspase activity also increased when the osteoblasts were co-cultured with the metastatic breast cancer cells (data not shown).

Apoptosis is Induced by Breast Cancer Cell Conditioned Medium

In order to determine if the apoptosis-inducing activity of the breast cancer cells was

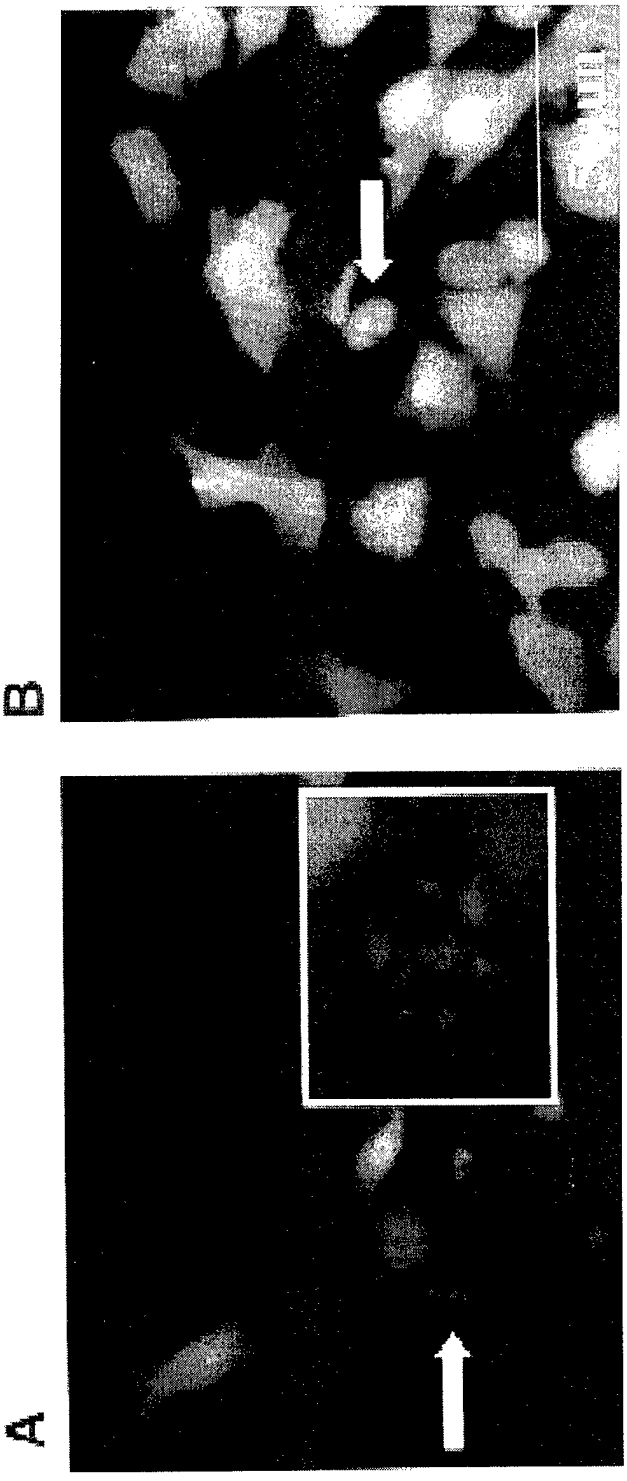

Fig. 1. Apoptotic nuclei in hFOB1.19 cells cultured with MDA-MB-435 or MDA-MB-231 cells. MDA-MB-435 cells (**A**) or MDA-MB-231 cells (**B**) expressing GFP were added to a confluent monolayer of hFOB1.19 cells (39°C) at a ratio of 1:100. After 48 h of co-culture the cells were stained with propidium iodide to detect dead cells, and immediately viewed with a fluorescence microscope. The arrows point to a fragmented nucleus (A) or condensed nucleus (B) indicative of apoptotic cells.

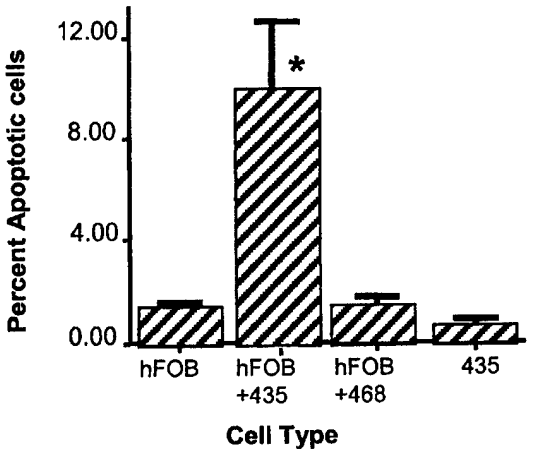

Fig. 2. Percentage of apoptotic hFOB1.19 cells in co-culture with breast cancer cells. Co-cultures of MDA-MB-435 or MDA-MB-468 cells expressing GPF were carried out as described in the legend to Figure 1. The cultures were fixed with a 2% paraformaldehyde and prepared for TUNEL assay of apoptotic nuclei using Cy5-dUTP [Jewell and Mastro, 2002]. Sytox® Orange was used as a counterstain. The cells were viewed with an Olympus BX-60 widefield digital microscope. A total of 700 fields were photographed for later analysis of TUNEL positive, GFP negative (hFOB1.19) and TUNEL positive, GFP positive (cancer cells). The percentages of apoptotic cells were calculated from the total number of cells on the coverslip. The experiment was done in triplicate and repeated. Shown are the mean ± SD. $*P < 0.01$ comparing hFOB cells plus MDA-MB-435 cells with hFOB cells alone.

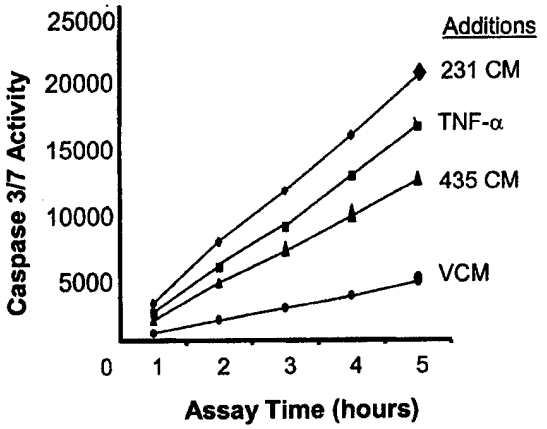

Fig. 3. Caspase activity in hFOB1.19 cells treated with breast cancer cell conditioned medium. The hFOB1.19 cells were plated at 1×10^6 per T 75 cm^2 tissue culture flask. Conditioned medium from the breast cancer cells was mixed 1:1 with normal differentiation culture medium and added to confluent, differentiated cultures of hFOBs and allowed to remain for 2 days at 39°C. Vehicle conditioned medium (VCM) was used for comparison. One set of samples was incubated with normal growth medium plus 0.3 ng/ml of TNF-α. After 48 h of incubation the cells were harvested and assayed for caspase 3/7 as described in the "Materials and Methods" section. The samples were assayed in duplicate; shown are the average values. The conditioned medium from MDA-MB-231 cells was tested seven times; from MDA-MB-435 cells three times, and TNF-α, six times with similar results.

relayed through a soluble factor, conditioned medium from the breast cancer cells lines was tested. Conditioned medium was diluted 1:1 with normal differentiation culture medium and added to confluent monolayers of hFOB1.19 cells. A vehicle control medium was mixed with culture medium as a control. After 48 h, caspase activity was assayed (Fig. 3). In this assay, conditioned medium from both MDA-MB-231 and MDA-MB-435 cells increased caspase activity two to fourfold over vehicle control medium (Fig. 3). On average there was about a twofold increase in caspase activity in the presence of conditioned medium from breast cancer cells (Table II) equivalent to treatment with 0.3 ng/ml TNF-α used as a positive control. In other

TABLE I. Quantitation of Apoptotic Osteoblasts by TUNEL Assay and Flow Cytometry

Treatment with media	Percent TUNEL positive cells	No. of expts.
[a]Breast cancer cell	4.5 ± 1.73	(6)
[b]Vehicle control	0.6 ± 0.3	(7)

The hFOB1.19 cells were treated with conditioned medium for 48 h as described in the legend to Figures 1 and 2 and the "Materials and Methods" section. The TUNEL protocol for flow cytometry (Promega) was used. Shown are the mean ± standard deviations. The number of experiments are given in parentheses. For comparison, treatment with TNF-α, 0.3 ng/ml, resulted in 5.6% TUNEL positive cells.
[a]Conditioned medium was 3 day MDA-MB-231 conditioned medium.
[b]Vehicle conditioned medium was the same as cell conditioned medium except it was not exposed to cells.

TABLE II. Caspase Activity of Osteoblasts in the Presence of Breast Cancer Conditioned Medium or TNF-α

Additions to hFOB1.19	Fold increase in caspase activity	No. of expts.
TNF-α	2.1 ± 0.4	(6)
Conditioned medium (231)	2.2 ± 0.50	(7)
Conditioned medium (435)	2.5 ± 0.2	(3)

Cells, hFOB1.19, were allowed to differentiate before they were treated with conditioned medium from MDA-MB-231 or MDA-MB-435 cells or with TNF-α (0.3 ng/ml) for 72 h. Caspase activity was assayed as described in the "Materials and Methods." Activity was compared to the values of cells incubated with vehicle conditioned medium. Shown are the average ± SD of activities. The number of experiments is given in parentheses.

experiments we determined that conditioned medium from 3T3 fibroblasts did not cause apoptosis (data not shown).

Fas is Expressed by hFOB1.19 Cells

It is reported that fas is expressed by primary cultures of osteoblasts [Kawakami et al., 1997] and that fas-ligand is expressed by breast cancer cells [Mullauer et al., 2000] and MDA-231 cells in particular [Keane et al., 1996]. Therefore, the interaction of fas with fas-ligand would seem a likely mechanism for breast cancer cell mediated apoptosis of hFOB cells. We also determined that the MDA-231 cells expressed fas-ligand mRNA. In order to ascertain that hFOB1.19 cells expressed fas, a non-cross linking biotin conjugated antibody to CD95 (anti-fas) and streptavidin RPE-Cy5 were used for flow cytometric analysis.

We found that fas was expressed both by proliferating and differentiated hFOB cells (Fig. 4). Unstained cells, cells incubated with an isotype control or cells incubated with streptavidin, but without biotinylated CD95 all showed the same low amount of background staining (Fig. 4). The breast cancer cells treated under the same conditions were negative for fas expression (data not shown). The differentiated cells always expressed greater levels of fas than the proliferating cells. The increase in fas expression in the differentiated cells versus the growing ones ranged from 2.3 to 44-fold (average of 16 within 5 experiments) depending on the length of time in differentiation medium.

Although the hFOB1.19 cells expressed fas, we considered that fas may be unable to transmit the death signal. To test this possibility, hFOB cells were incubated with cross-linking IgM CD95 alone or together with cycloheximide, an inhibitor of protein translation which sensitizes cells to fas-induced apoptosis [Mullauer et al., 2000]. CD95 alone caused about 17% apoptosis; while CD95 plus cycloheximide increased this value to about 25% when compared to cells treated with cycloheximide alone (Fig. 5). Cycloheximide consistently slightly decreased apoptosis (>100% viability) compared to cells with no treatment.

A fas-ligand over-expressing cell line, WR19L-A12 [Tanaka et al., 1998] was used to determine the sensitivity of fas expressing hFOB1.19 cells to fas-ligand induced cell death. Co-culture of hFOB1.19 cells with WR19L-A12

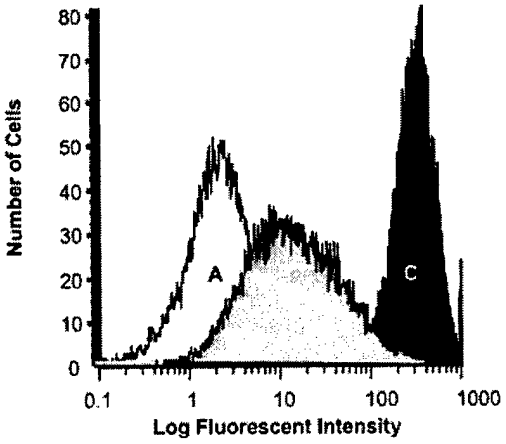

Fig. 4. Expression of fas by hFOB1.19 cells. The hFOB1.19 cells were grown at 34°C until confluent. Proliferating cells were kept at 34°C in growth medium. The other cells were switched to differentiation medium and cultured for 2 more days at 34°C. The cells were removed from the plate with Accutase™ (Phoenix Flow Systems, San Diego, CA), resuspended and washed in staining solution (5% calf serum, 2.5% goat serum, 0.1% NaN_3 in complete PBS). The cells were incubated for 30 min at 4°C in the dark with biotin conjugated anti-fas (CD95, Pharmingen, Bedford, MA) 10 µl/10^6 cells; washed and resuspended with steptavidin RPE-CY5 (DAKO) 1/10 dilution (10 µl) for 20 min, 4°C, in the dark; washed and either analyzed immediately with the flow cytometer (Coulter XL-MCL) or fixed (0.2% paraformaldehyde in PBS) and analyzed the next day. An isotype matched antibody was used as a control. **A**: Cells with isotype control antibody; **B**: Proliferating cells; **C**: Differentiating cells.

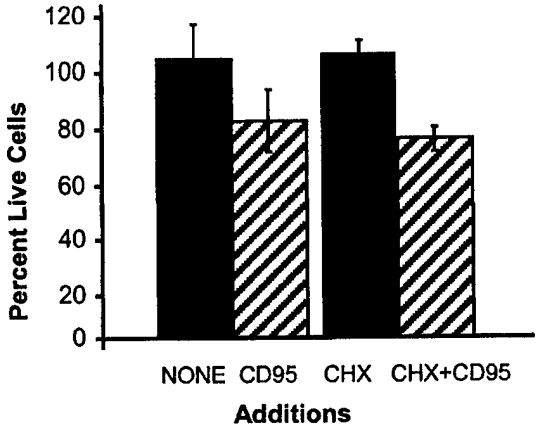

Fig. 5. Induction of hFOB1.19 cell death by anti-fas (CD95) antibody. The hFOB1.19 cells (5.4×10^4 cells) were grown in 24-well plates at 34°C until 80% confluent (about 3 days), changed to differentiation medium, and incubated at 39°C for 2 more days. Cycloheximide (10 ng/ml) and/or CD95 (anti-CD95 IgM, CH-11 Immunotech Marseille, Cedex, France) (100 ng/ml) were added and the cells incubated for an additional 48 h. Viable cells were assayed by the MTT assay following the protocol provided by Sigma. Data are expressed as percentage live cells compared to cultures with no treatment. The assay was done in duplicate and variance is indicated by the bars.

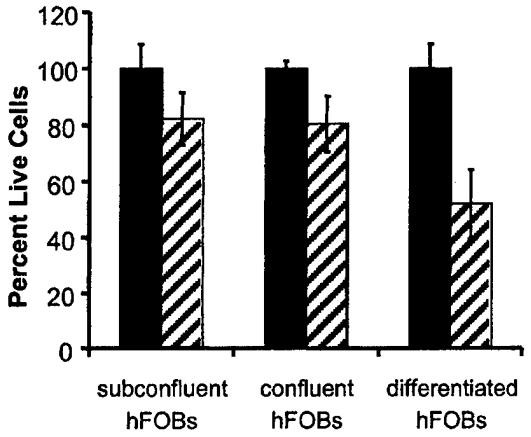

Fig. 6. Death of hFOB1.19 cells induced by fas-ligand over-expressing WR19L-A12 cells. The h-FOB1.19 cells were plated in growth medium at 5×10^3/well or 1×10^4/well in 24-well plates. After the cells plated at 1×10^4 became confluent, one half of the wells were incubated for 2 more days in differentiation medium at 39°C (differentiating) or at 34°C in regular growth medium (confluent). The cells plated at 5×10^3/well were treated when subconfluent, with either cycloheximide alone (10 ng/ml) (solid bars) or cycloheximide plus 5×10^4 WR19L-A12 cells (striped bars). After 48 h the media were removed and all the plates washed with PBS to remove non-adherent cells. The viable, attached cells, were assayed according to the MTT protocol. The experiment was carried out with triplicate samples. Shown are the average values compared to cultures without treatment.

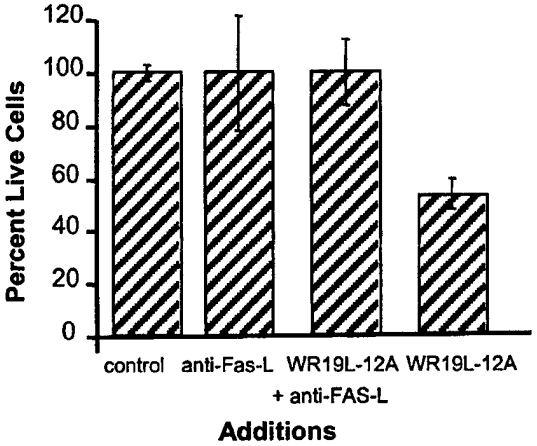

Fig. 7. Fas-ligand induced apoptosis in hFOB1.19 cells. The hFOB1.19 cells were plated at 5×10^4/well in 24-well plates in growth medium and incubated at 34°C for 3 days. The medium was changed to differentiation medium and to 39°C at 3 days when the cultures were nearly confluent. After 2 days, WR19L-A12 cells were added at 5×10^4/well to some wells. In one case the WR19L-A12 cells were incubated with anti-fas-ligand antibody (NOK-1, BD Pharmingen, 1 μg/ml) for 15 min before addition to the hFOBs. The co-cultures were incubated at 39°C for 48 h; the medium removed; and all plates washed with PBS. Remaining viable cells were assayed by the MTT assay. Shown are the average of duplicate samples with the variance.

cells for 2 days resulted in up to about 50% cell death (Fig. 6). As predicted from the data shown in Figure 4, the differentiated hFOB were more sensitive than growing or even confluent but non-differentiated cells. Cycloheximide alone did not cause apoptosis and did not enhance fas-ligand induced apoptosis by these cells.

To ascertain that the apoptosis induced by the WR19L-A12 cells was due to fas-ligand, a blocking antibody to fas-ligand was added. It prevented the WR19L-A12 cell-induced apoptosis (Fig. 7).

In order to compare the levels of fas-ligand expressed by the MDA-MB-231 and MDA-MB-435 cells with the WR19L-A12 cells, we carried out flow cytometry using a biotin conjugated, anti-fas-ligand monoclonal antibody and RPE-Streptavidin. The WR19L-A12 cells were clearly positive compared to isotype controls, but the breast cancer cells were not (data not shown). Because cancer cells often express metalloproteinase activity which may cleave fas-ligand [Kayagaki et al., 1995], they were incubated for 48 h with a metalloproteinase inhibitor, GM-6001. Nonetheless, expression of cell surface fas-ligand was not detected.

RNA was collected from the breast cancer cell lines and RTPCR was carried out to determine if fas-ligand mRNA were expressed. We detected fas-ligand mRNA in the MD-MBA-231 cells, but we were unable to detect it in the MD-MBA-435 cells (data not shown).

TNF-α Causes Apoptosis of hFOB1.19 Cells

It has been reported that human osteoblasts were sensitive to fas-induced death in the presence of TNF-α, apparently because TNF-α up-regulates fas [Tsuboi et al., 1999]. The same was true for hFOB1.19 cells. When these cells were incubated with 0.3 ng/ml TNF-α for 48 h they showed a dramatic increase in fas expression (Fig. 8). In addition it was seen that TNF-α alone induced cell death in hFOB1.19 cells (Fig. 9). Death was mediated by caspases (Fig. 10) and thus likely to occur by apoptosis. This TNF-α induced cell death was reversed by a blocking antibody to TNF-α (Fig. 11).

Conditioned medium from both MDA-MBA-231 cells and MDA-MBA-435 cells were tested by ELISA for the presence of TNF-α. None was detected at the level of 10 pgm/ml.

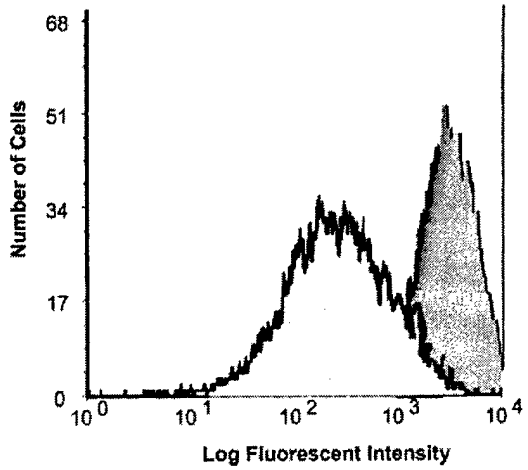

Fig. 8. Up-regulation of fas expression in hFOB1.19 cells by TNF-α. hFOB1.19 cells were plated at 5×10^5 in T 25 cm^2 flasks and incubated at 34°C overnight. The next morning the medium was changed to differentiation medium and cultures incubated at 39°C. At this time TNF-α (0.3 ng/ml) was added and 48 h later the cells were harvested and analyzed for fas expression by flow cytometry and biotin conjugated anti-fas as described in the "Materials and Methods" section and in the legend to Figure 4.

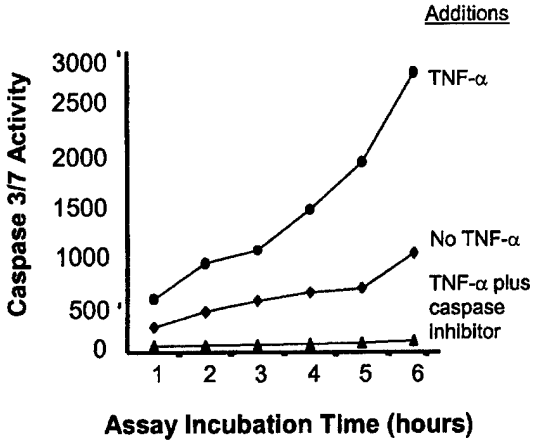

Fig. 10. Caspase activity following TNF-α treatment of hFOB1.19 cells for 48 h. hFOB1.19 cells were plated at 10^6 cells per T 75 cm^2 tissue culture flasks. The cells were allowed to differentiate at 39°C; TNF-α (3 ng/ml) was added and the flasks were incubated for 48 h more. To one flask 0.025 nM caspase inhibitor (Ac-DEVD-CHO) was added. Shown is one experiment out of five.

DISCUSSION

Osteolytic bone diseases including metastatic breast cancer disrupt the normal skeletal equilibrium so that bone formation and bone resorption are no longer coupled. As metastatic breast cancer progresses, there is severe loss of bone. Understanding exactly how that balance is upset is critical for devising methods for restoring it. It is clear that osteoclast activity increases probably due to increased osteoclastogenesis, increased osteoclast activity, and

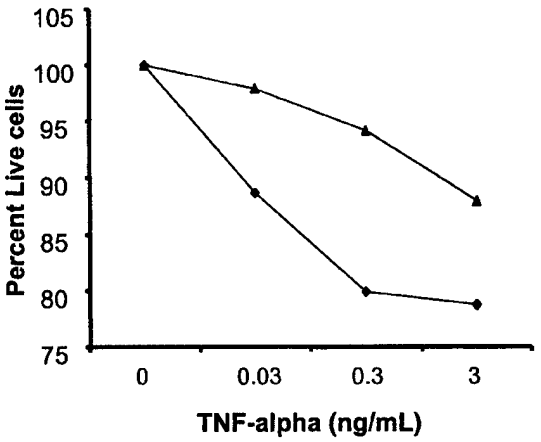

Fig. 9. Induction of apoptosis by TNF-α of hFOB1.19 cells. hFOB1.19 cells were plated at 5×10^3 well in 96-well plates and incubated at 34°C overnight. The medium in one group was then changed to differentiation medium (triangles) and cultures incubated at 39°C. The medium was replaced with growth medium (squares) in the second group and cultures were incubated at 34°C. TNF-α was added over a range of concentrations as indicated. After 48 h the cells were assayed for viability using Cell Titer 96® Aqueous Assay (Promega). Shown are the averages of triplicate samples. The standard deviations were all <0.06%.

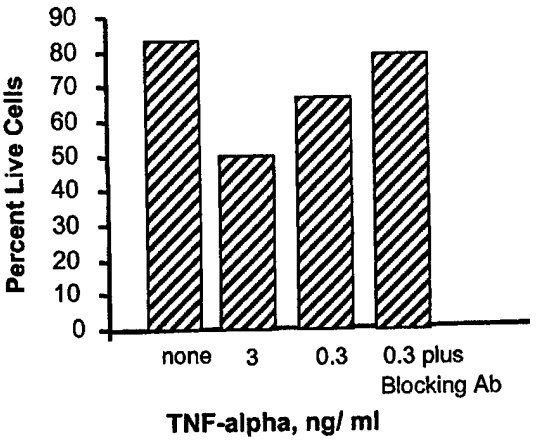

Fig. 11. Reversal of TNF-α mediated cell death with a blocking antibody to TNF-α. The hFOB1.19 cells were plated at 5×10^5 in T 25 cm^2 flasks and incubated at 34°C overnight. The next day TNF-α, pre-incubated for 10 min with neutralizing antibody to TNF-α (15 μg/ml) was added to one flask. The remaining cultures received TNF-α at two concentrations as indicated. Cells were incubated for further 48 h. Viable cells were assayed by trypan blue exclusion.

increased life span [Thomas et al., 1999]. However, we believe that the osteoblast also contributes to the imbalance.

The data presented in this study indicate that osteolytic, metastatic breast cancer cells increased the prevalence of apoptosis in a human osteoblast cell line, hFOB1.19. Apoptosis was detected by several mechanisms including TUNEL and caspase activity. Quantitation indicated that the percentage of apoptotic osteoblasts increased several fold, from about 2% to an average of 10%, in the presence of metastatic breast cancer cells or their medium. This finding is consistent with other studies of diseases or conditions that lead to bone loss such as osteoporosis [Clohisy and Ramnaraine, 1998; Clohisy, 1999], rheumatoid arthritis [Tsuboi et al., 1999; Manolagas, 2000], glucocorticoid-induced bone loss [Weinstein et al., 1998], and *Staphylococcus aureus* infection [Alexander et al., 2001]. To consider one example, glucocorticoid-induced bone loss is associated with a change in osteoblast apoptosis in vivo from about 0.7 to about 2% [Weinstein et al., 1998]. This change combined with decrease osteoblastogenesis and increased osteoclast activity is sufficient to account for the observed overall bone loss.

The skeleton is a very dynamic organ. During development, the skeleton is modeled, i.e., bone is deposited and removed in order for the skeleton to achieve its mature structure. However, the mature skeleton continues to be replaced [Manolagas, 2000]. This remodeling process that takes place in normal bone as well as damaged bone. It is estimated that the skeleton regenerates itself every 10 years [Parfitt, 1994]. Because remodeling involves a complicated interplay between osteoblasts and osteoclasts, lack of coordination results in too much bone deposition (osteopetrosis) or too much bone loss (e.g., osteopetrosis, osteoporosis). After the relatively short-lived osteoclasts degrade bone, the osteoblasts are recruited to repair it. Osteoprogenitor cells proliferate and differentiate into mature osteoblasts which produce the matrix for mineralization. At the end of this process an estimated 30–50% of the osteoblasts remain as bone lining cells or become trapped in the matrix as osteocytes. The remaining 50–70% undergo apoptosis [Jilka et al., 1998]. Apoptosis is a rapid process and there is evidence that in vivo the apoptotic osteoblasts are quickly disposed of [Cerri et al., 2003]. Nevertheless apoptotic osteoblasts can be detected under conditions of excessive loss [Jilka et al., 1998; Weinstein et al., 1998] or rapid turnover [Cerri et al., 2003]. In addition, in vitro analysis of osteoblast proliferation supports the idea that apoptosis occurs as part of the normal life cycle.

In osteolytic breast cancer metastasis the role of apoptosis of osteoblasts has not been previously investigated [Mastro et al., 2003]. Activated osteoblasts are important intermediaries in the model in which PTHrP produced by tumor cells leads to osteoclast activation. The PTHrP causes increased RANKL expression, but down-regulates OPG produced by osteoblasts. The osteoblasts in turn cause osteoclast activation. While this activation may occur in humans as part of the metastatic process, there is also clinical evidence of osteoblast loss at sites near the tumors [Stewart et al., 1982]. We suggest that osteoblasts undergo apoptosis in the presence of breast cancer cells, and loss by apoptosis must be considered in the overall equation.

What is the mechanism by which osteoblast apoptosis is induced? The specific initiators of osteoblast apoptosis under various physiological conditions are not well known. The literature suggests several initiators of osteoblast apoptosis under inflammatory conditions or during normal bone remodeling. In vitro studies indicate the necessity for fas–fas ligand interactions [Kawakami et al., 1997] in human osteoblasts apoptosis. Primary osteoblasts normally express fas. It is not clear what cells display fas-ligand. Under conditions of inflammation, activated T-cells could play this role and participate in inflammatory bone loss. Because many cancer cells including MDA-MB-231 cells have been reported to express fas-L [Keane et al., 1996; Mullauer et al., 2000] it seemed likely that the fas–fas ligand pathway is involved in breast cancer mediated osteoblast apoptosis. In order to test this, we determined that hFOB1.19 cells expressed fas; expression was greater in the more differentiated compared with less differentiated cells; and apoptosis was induced by fas-ligand over-expressing cells or through cross linking of fas by anti-fas antibody. Nevertheless we were unable to demonstrate that the cancer cells expressed fas-ligand by flow cytometry. It is known that fas-ligand can be rapidly cleaved from the surface with metalloproteinases. This is consis-

tent with our inability to detect fas-ligand with flow cytometry. Further examination by RT-PCR for the mRNA for fas-L was negative in the MDA-MB-435 cells although it was expressed in MDA-MB-231 cells. Taken together the data suggests that in this model fas-ligand is not the initiator of apoptosis.

TNF-α also has been linked to osteoblast apoptosis in vivo [Kimble et al., 1997] and in vitro [Tsuboi et al., 1999]. In this present study, we saw that the hFOB cells were susceptible to TNF-α induced apoptosis (Fig. 10). TNF-α also up-regulated fas expression in hFOB cells as previously reported for primary osteoblasts and other osteoblast lines [Tsuboi et al., 1999]. Pederson et al. [1999] report that MDA-MB-231 cells secrete TNF-α, but we were unable to detect it in the conditioned medium of either MDA-MB-231 or of MDA-MB-435 cells by ELISA. The difference may be that Pederson et al. [1999] collected three-day conditioned medium and fractionated it prior to testing, whereas we used 24 h unfractionated conditioned medium. It also is likely that in vivo, under conditions of metastatic growth where there is an inflammatory response, cytokines such as TNF-α or IL-6 would be produced by immune cells at the site.

There are other postulated inducers of osteoblast apoptosis. Apoptosis is part of the normal bone remodeling process so matrix degradation products may play a role. Inorganic phosphate [Adams et al., 2001] released from bone and RGD sequences from fibronectin and osteopontin are potent apoptogens in vitro [Perlot et al., 2002]. OPN is produced by the cancer cell lines, but without a method to detect or block such proposed apoptogens, the hypothesis remains untested.

Alexander et al. [2001] reported the up-regulation of TRAIL in bone in the presence of *Staphylococcus aureus*. We also found TRAIL expression in hFOB cells using RTPCR (data not shown). However, an inhibitor of TRAIL, DR4, did not prevent metastatic cell induced apoptosis of the hFOB cells. Further, breast cancer cell conditioned media did not up-regulate TRAIL expression by the osteoblasts. Therefore, TRAIL does not seem to be important in this case.

Other reported possibilities include bone morphogenetic protein-2 (BMP-2) [Hay et al., 2001], oxygen radicals under inflammatory conditions [Kelpke et al., 2001], or fibroblast growth factor FGF 2 [Mansukhani et al., 2000].

Fromigue et al. [2001] using a stromal cell line showed that MCF-7 breast cancer cells secrete factors that brought about osteoblast apoptosis and necrosis. However, MCF-7 cells are weakly invasive and do not normally metastasize to bone [Thomas et al., 1999]. Saunders et al. [2001] also reported that metastatic MDA-MB-435 cells form heterotypic gap junctional communication with hFOBs. Whether gap junction disruption is associated with the apoptotic process is not clear. There are many possible mechanisms for initiation of osteoblast apoptosis both in the normal remodeling process and in disease states. At this time we do not know which contribute to bone loss by osteolytic breast cancer metastases, but it is clear that breast cancer cells cause abnormally high rates of apoptosis in osteoblasts.

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Breast Cancer Metastasis Suppressor 1 (BRMS1) Forms Complexes with Retinoblastoma-binding Protein 1 (RBP1) and the mSin3 Histone Deacetylase Complex and Represses Transcription*

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Breast cancer metastasis suppressor 1 (BRMS1) suppresses metastasis of multiple human and murine cancer cells without inhibiting tumorigenicity. By yeast two-hybrid and co-immunoprecipitation, BRMS1 interacts with retinoblastoma binding protein 1 and at least seven members of the mSin3 histone deacetylase (HDAC) complex in human breast and melanoma cell lines. BRMS1 co-immunoprecipitates enzymatically active HDAC proteins and represses transcription when recruited to a Gal4 promoter *in vivo*. BRMS1 exists in large mSin3 complex(es) of ~1.4–1.9 MDa, but also forms smaller complexes with HDAC1. Deletion analyses show that the carboxyl-terminal 42 amino acids of BRMS1 are not critical for interaction with much of the mSin3 complex and that BRMS1 appears to have more than one binding point to the complex. These results further show that BRMS1 may participate in transcriptional regulation *via* interaction with the mSin3·HDAC complex and suggest a novel mechanism by which BRMS1 might suppress cancer metastasis.

The complex process of cancer cell dissemination and the establishment of secondary foci involves the acquisition of multiple abilities by metastatic cells. For example, blood-borne metastasis requires cells to invade from the primary tumor, enter the circulation, survive transport, arrest at a secondary site, recruit a blood supply, and proliferate at that site (1). The ability to accomplish all of these steps likely involves changes in, and coordinated expression of, a large assortment of genes. Consistent with this notion, several genes, proteins, and pathways have been associated with metastatic progression, including oncogenes, motility factors, and matrix metalloproteinases (1, 2). In addition to metastasis-promoting genes, a new class of molecules called metastasis suppressors has been described (reviewed in Refs. 2–5). By definition, metastasis suppressors inhibit metastasis without blocking primary tumor growth, presumably by inhibiting one or more steps necessary for metastasis. To date, 13 metastasis suppressor genes have been identified that reduce the metastatic ability of cancer cell line(s) *in vivo* without affecting tumorigenicity, namely breast cancer metastasis suppressor 1 (BRMS1),[1] CRSP3, DRG1, KAI1, KISS1, MKK4, NM23, RhoGDI2, RKIP, SSeCKs, VDUP1, E-cadherin, and TIMPs (reviewed in Refs. 4 and 5).

We identified *BRMS1* using differential display to compare highly metastatic breast carcinoma cells with related but metastasis-suppressed cells (6). Enforced expression of BRMS1 suppressed metastasis in three animal models, namely human breast (6), murine mammary (7), and human melanoma cells (8). Additionally, *BRMS1* mapped to loci in murine (7) and human (6) genomes that had previously been implicated in metastasis control (9). The BRMS1 protein localized to nuclei and restored gap junctional intercellular communication in both breast and melanoma tumor cell lines (8, 10, 11), but its molecular functions remain to be elucidated.

One approach to determine a mechanism of action involves identifying which proteins interact with BRMS1. In this report, we utilized yeast two-hybrid and co-immunoprecipitation (co-IP) to demonstrate that BRMS1 interacts with retinoblastoma-binding protein 1 (RBP1). This association led to experiments to demonstrate that BRMS1 interacts with at least seven members of the mammalian Sin3 (mSin3) mSin3·histone deacetylase (HDAC) complexes, including HDAC1 and HDAC2.

Human HDACs exist in many large, multi-subunit protein complexes (12) that are recruited to specific regions by DNA-binding factors. As their name indicates, HDACs remove acetyl groups from lysine residues at the N-terminal tails of core

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) XM_045014.

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[1] The abbreviations used are: BRMS1, breast cancer metastasis suppressor gene 1; co-IP, coimmunoprecipitation; RBP1, retinoblastoma binding protein 1; HDAC, histone deacetylase; mSin3, mammalian Sin3 (suppressor of defective silencing 3); NuRD, nucleosomal remodeling and deacetylation; Rb, retinoblastoma; NCoR, nuclear receptor corepressor; SMRT, silencing mediator for retinoic acids and thyroid hormone receptor.

histones (13–15). Histone deacetylation favors transcriptional repression, whereas acetylation (mediated by histone acetyltransferases) favors transcriptional activation. mSin3·HDAC complexes are named for the large mSin3A and mSin3B proteins, which are thought to serve as scaffolds for complex assembly (14). HDAC enzymatic activity in mSin3 complexes is mediated by a core subunit consisting of HDAC1, HDAC2, RbAp46, and RbAp48 (13). The core HDAC subunit is also found in at least one other HDAC complex, NuRD (nucleosomal remodeling and deacetylation (16).

Mammalian Sds3 (mSds3; suppressor of defective silencing 3) was recently reported to be an integral component of the mSin3 complex and acts to stabilize HDAC1 within the complex (17). BRMS1 shares homology with mSds3, suggesting that BRMS1 belongs to a protein family (17). mSin3-associated proteins, SAP18 and SAP30, which are believed to serve as adapter molecules, complete the core complex as currently understood (18–20).

MATERIALS AND METHODS

Cell Lines, Cell Culture, and Transfections—MDA-MB-231 is a human estrogen receptor- and progesterone receptor-negative cell line derived from a pleural effusion from an infiltrating ductal breast carcinoma. C8161 is a metastatic, amelanotic human melanoma cell line derived from an abdominal wall metastasis. C8161.9 is a highly metastatic clone obtained by limiting dilution cloning of C8161 (21). 66cl4 is a murine mammary carcinoma cell line derived from a spontaneous carcinoma in BALB/cfC3H mice (22, 23). All cell lines were cultured in a 1:1 mixture of Dulbecco's modified minimum essential medium and Ham's F12 medium supplemented with 5% fetal bovine serum (Atlanta Biologicals, Atlanta, Georgia), 1% non-essential amino acids, and 1 mM sodium pyruvate. Transfected cells also received 500 μg/ml G418 (Geneticin; Invitrogen). All cells were maintained on 100-mm Corning tissue culture dishes at 37 °C with 5% CO_2 in a humidified atmosphere. MDA-MB-231 cells were passaged at 80–90% confluence using a solution of 0.125% trypsin and 2 mM EDTA in Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate buffered saline (CMF-DPBS). C8161.9 and 66cl4 cells were passaged at 80–90% confluence using 2 mM EDTA in CMF-DPBS. BRMS1 was cloned into the constitutive mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA) under control of the cytomegalovirus promoter. No antibiotics or antimycotics were used. All cell lines were found to be negative for *Mycoplasma* spp. contamination using a PCR-based method (TaKaRa, Madison, WI).

To detect BRMS1 protein expression, a chimeric molecule was constructed with an N-terminal epitope tag (SV40T epitope 901) (24, 25). Epitope-tagged full-length BRMS1 and deletion mutants were cloned into pcDNA3 before introduction into cells by electroporation (Bio-Rad model Gene Pulser; 220 V, 960 microfarads, ∞ ohms). Briefly, cells (0.8 ml; 1×10^7 cells/ml) from 80% confluent plates were detached, plasmid DNA (10–40 μg) was added to the cells, and the mixture was placed onto ice for 5 min before electroporation, followed by 10 min on ice prior to plating on 100-mm cell culture dishes. Transfectants were selected using G418 (Geneticin; 500 μg/ml). Single-cell clones were isolated by limiting dilution in 96-well plates. Stable transfectants were assessed for protein expression by immunoblotting.

Constructs—Deletion mutants were created by unidirectional digestion with exonuclease III as described previously (26). Briefly, pcDNA3 901-BRMS1 was digested by ApaI and Bsu36I in the 3′ multiple cloning site and then digested with 150 units/pmol DNA exonuclease III (Promega) at 37 °C. Reactions were stopped at different time points to create a nested set of C-terminal BRMS1 deletion mutants. Sequencing confirmed that the following 3′ deletion mutants were successfully created: 1) 901-BRMS1(Δ204–246) + LFYSVT; 2) 901-BRMS1(Δ164–246) + TIL; and 3) 901-BRMS1(Δ91–246) + FYSVT. Additional amino acids were added because a short stretch of vector DNA was transcribed prior to encountering a stop codon. Hereafter, these constructs will be designated BRMS1(Δ204–246), BRMS1(Δ164–246), and BRMS1(Δ91–246), respectively.

Antibodies—An antibody directed against the 901 epitope was generously provided by Dr. Satvir Tevethia. Anti-MTA1 was a gift from Dr. Garth Nicolson. Anti-RBP1 (clone LY32 and initial aliquots of clone LY11) were gifts of Dr. Philip Branton. Antibodies directed against HDAC1, HDAC3, NCoR, RBP1 (clone LY11), SAP30, mSin3A, and SMRT were purchased from Upstate Biotechnology (Lake Placid, NY). Antibodies recognizing E2F and retinoblastoma (Rb) were bought from Pharmingen. Antibodies directed against HDAC2, Mad1, Max, Mi-2, p107, p130, RbAp46, RbAp48, SAP18, and mSin3B were obtained from Santa Cruz Biotechnology.

Yeast Two-hybrid Screen—A yeast two-hybrid screen was performed to isolate cDNAs encoding BRMS1-interacting proteins essentially as described in the manufacturer's instructions (Clontech MATCHMAKER LexA). Full-length BRMS1 was cloned in-frame with the GAL4 DNA binding domain in the pDBTrp (Invitrogen) vector to obtain pDB-BRMS1. This GAL4DB-BRMS1 fusion (bait) construct was used to transform AH 109 (*MATa, trp-901, leu2–3, 112, ura3–52, his3–200, gal4Δ, gal80Δ, LYS2::*GAL_{UAS}*-GAL1*$_{TATA}$*-HIS3, GAL2*$_{UAS}$*-GAL2*$_{TATA}$*-ADE2, URA3::MEL1*$_{UAS}$*-MEL1*$_{TATA}$*-lacZ, MEL1*). Human breast, prostate, and placenta cDNA libraries in pACT2 (MATCHMAKER, BD Biosciences Clontech) were screened in yeast drop-out minimal medium lacking histidine, tryptophan, and leucine. His^+ colonies were tested for growth on minimal medium lacking adenine, tryptophan, leucine, and β-galactosidase activity as described previously (27). cDNA plasmids were isolated from each positive yeast clone using Zymoprep (Zymo Research, Orange, CA) and sequenced. The interaction phenotype was lost when either the bait or prey plasmid was lost from the cell. Re-introduction of missing partners restored growth on minimal medium lacking histidine, tryptophan, and leucine, growth on medium lacking adenine, tryptophan and leucine, and restoration of β-galactosidase activity.

^{35}S Protein Labeling—Cells were grown to 80–90% confluence in 100-mm tissue culture plates. Media were removed and replaced with 3 ml of cysteine-methionine-free media (Invitrogen) containing 5% fetal bovine serum for 1 h. Media were removed and replaced with 3 ml of cysteine- and methionine-free media containing 5% fetal bovine serum and 100 μCi/ml ^{35}S-express protein labeling mix (PerkinElmer Life Sciences). Cells were incubated for 18 h before protein was collected for co-IP.

Co-immunoprecipitation—Cells (90–95% confluence) were washed twice with ice-cold PBS and lysed with ice-cold lysis buffer (0.5% Igepal CA-630 (Sigma), 50 mM Tris, pH 8, 150 mM NaCl, and 2 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 50 mM NaF, 0.2 mM Na_3VO_4, and 10 μl/ml of a protease inhibitor mixture containing 4-(2-aminoethyl) benzensulfonylfluoride (AEBSF), pepstatin A, *trans*-epoxysuccinyl-L-leucylamido(4-guanido)butane (E-64), bestatin, leupeptin, and aprotinin (Sigma). Lysate was kept at 4 °C during all subsequent steps. Lysate was passed through a 21-gauge needle several times, incubated on ice for 1 h, then centrifuged for 1 h at 12,000 × *g* in a Sorvall MC 12V microcentrifuge with an F12/M.18 rotor to remove insoluble debris. Lysates were then rocked gently in the presence of antibody for 1 h, followed by the addition of 20 μl of protein A/G PLUS agarose beads (Santa Cruz Biotechnology) and rocking overnight. Agarose beads were washed twice with ice-cold PBS, heated to 60 °C in sample buffer, subjected to SDS-PAGE, and transferred to polyvinylidene difluoride membrane for immunoblotting. For ^{35}S-labeled samples, films were exposed directly to polyvinylidene difluoride membranes. In each experiment, blots were probed with antisera to the immunoprecipitated protein to verify the quality of the immunoprecipitation and assess the equal loading of lanes.

Size Exclusion Chromatography—Whole cell protein lysate (pooled from 10 100-mm plates using 1 ml of lysis buffer each) was applied to a Superose 6 HR 10/30 size exclusion column (Amersham Biosciences). The column was run using lysis buffer with 1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol at a flow rate of 0.2 ml/min. Fractions (500 μl) were collected, and 420 μl of each fraction were used for co-IP. The remaining 80 μl was used for immunoblotting.

HDAC Activity Assay—Following co-IP, agarose beads were combined with 400 μl of HDAC assay buffer (15 mM Tris, pH 7.9, 10 mM NH_4Cl, 0.25 mM EDTA, 10% glycerol, and 10 mM β-mercaptoethanol) containing 1.5 μg ^3H-labeled chicken reticulocyte core histones (28) with or without 250 mM sodium butyrate (an HDAC inhibitor). Samples were inverted continuously on a rotating wheel for 3 h at 30 °C, and HDAC activity was measured as described previously (28). Briefly, the reaction was stopped by adding 100 μl of 1 M HCl/0.4 M acetic acid and 0.8 ml ethyl acetate. Samples were vortexed for 30 s and centrifuged at 8,000 × *g* for 5 min. An aliquot (0.6 ml) of the upper (organic) phase was then counted for radioactivity in a 5-ml scintillation mixture (Fisher).

Reporter Assays—BRMS1 cDNA was cloned in-frame with the N-terminal Gal4-DNA binding domain in pBIND (Promega). Subconfluent (80–90%) COS7 cells were transfected using the FuGENE reagent (Roche Diagnostics) with the GAL4-BRMS1 fusion construct and a luciferase reporter plasmid containing four GAL4 binding sites upstream of the myelomonocytic growth factor minimal promoter, kindly provided by Dr. Ron Eisenman. pRLSV40 (*Renilla* luciferase) was used as a transfection control. Trichostatin A (50, 150, and 300 ng/ml, Sigma)

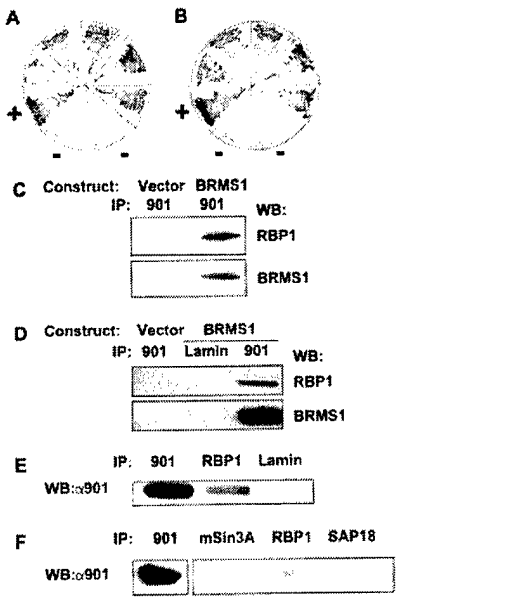

FIG. 1. **Yeast two-hybrid and co-IP indicate that BRMS1 interacts with RBP1.** *A*, growth of representative positive candidates on minimal media lacking histidine, tryptophan, and leucine. *B*, growth of representative positive candidates on minimal medium lacking adenine, tryptophan, and leucine. *Plus sign* (+) indicates positive control, *minus sign* (−) indicates negative control (AH109 with BRMS1 or interactor cDNA alone). *C*, BRMS1 co-immunoprecipitated RBP1 from whole cell lysate (1 mg) in MDA-MB-231 cells. Anti-901 was used to immunoprecipitate (*IP*) epitope-tagged BRMS1 and also pulled down RBP1, as shown by Western blot (*WB*). Anti-901 did not pull down RBP1 in vector-transfected cells. *D*, BRMS1 co-immunoprecipitated RBP1 from whole cell lysate (1 mg) in C8161.9 cells. Anti-901 was used to immunoprecipitate epitope-tagged BRMS1 and also pulled down RBP1, as shown by Western blot. Anti-901 did not pull down RBP1 in vector-transfected cells, and anti-Lamin A/C (an irrelevant antibody) did not pull down RBP1 in BRMS1-transfected cells. *E*, anti-RBP1 co-immunoprecipitated BRMS1 in MDA-MB-231 cells. Immunoblotting with α-901 was used as a positive control, and α-Lamin A/C was used as a negative control. *F*, anti-RBP1 co-immunoprecipitated BRMS1 in C8161.9 cells. Immunoblotting with α-901 was used as a positive control, and α-mSin3A and α-SAP18 were used as negative controls.

was added for 24 h prior to lysis. Cells were lysed in Passive lysis buffer (Promega) 48 h post-transfection. Cell extracts were assayed for luciferase activity using the Dual luciferase reporter assay system (Promega) and an automated luminometer Monolight™3010 (Pharmingen). Transfection efficiencies were normalized using the *Renilla* luciferase control.

RESULTS

RBP1 and mSds3 Were Identified as BRMS1-interacting Proteins by Yeast Two-hybrid Screen—A yeast two-hybrid screen was performed using prey libraries from three human tissues, breast, placenta, and prostate. Breast was chosen because BRMS1 was first identified as a metastasis suppressor in breast cancer. Placenta and prostate were chosen because BRMS1 mRNA is highly expressed in these tissues (6). Full-length BRMS1 was used as the "bait." RBP1 was present in the majority of positive clones from breast and placenta libraries, so it was chosen for further studies (Fig. 1, *A* and *B*).

The FLJ00052 expressed tag was present as two independent positive clones in a prostate library screen. During the completion of the work reported here, FLJ00052 was identified as the mammalian ortholog (mSds3, GenBank™ accession number XM_045014 mapping to human chromosome 12q24.23) of the yeast Sds3 protein. There are other related genes according to the LocusLink (www.ncbi.nlm.nih.gov/LocusLink/list.cgi?Q = FLJ00052&ORG = &V = 0), suggesting the existence of additional mSds3 orthologs. mSds3 is an integral component of the mSin3·HDAC co-repressor complex, modulates HDAC activity, and stabilizes the complex (17). Antibodies recognizing mSds3 are not available commercially; thus, we have not yet been able to test whether BRMS1 pulled down mSds3.

BRMS1 and RBP1 Are Reciprocally Co-immunoprecipitated in Human Breast and Melanoma Cancer Cells—MDA-MB-231 human breast carcinoma cells and C8161.9 human melanoma cells were transfected with 901 epitope-tagged BRMS1. Immunoprecipitation of BRMS1 followed by immunoblot with two RBP1-specific antibodies (clones LY11 and LY32) (Fig. 1, *C* and *D*) showed that BRMS1 co-immunoprecipitates RBP1 (Fig. 1, *C* and *D*). Negative controls (co-IP using anti-901 in vector-transfected cells or co-IP using an irrelevant antibody, anti-Lamin A/C) did not pull down RBP1 (Fig 1, *C* and *D*). Antibody directed against RBP1 co-immunoprecipitated BRMS1 in both breast carcinoma (Fig. 1*E*) and melanoma (Fig. 1*F*) cells.

To begin defining the binding domains of BRMS1 responsible for interactions with RBP1, three C-terminal deletion mutants of 901-tagged BRMS1 were generated by exonuclease III digestion, designated BRMS1(Δ204–246), BRMS1(Δ164–246), and BRMS1(Δ91–246) (Fig. 2*C*). Deletion constructs were transfected into both MDA-MB-231 and C8161.9. The latter expressing clones were experimentally more useful, because expression of all three deletion mutants was approximately equivalent to full-length protein (data not shown, but can be inferred from Fig. 2*B*). In MDA-MB-231, only BRMS1(Δ204–246)-expressing clones had protein levels approximating full-length BRMS1 (inferred from Fig. 2*A*). Anti-901 antibody was used to co-immunoprecipitate deletion mutants, and immunoblotting was used to detect RBP1 (Fig. 2, *A* and *B*). Loss of amino acids 204–246 did not decrease binding to RBP1 in either cell line (Fig. 2, *A* and *B*). Loss of amino acids 164–246 diminished binding (by ~90% by densitometry), and loss of amino acids 91–246 abrogated binding (Fig. 2*B*). Absence of binding by BRMS1 (Δ91–246) was controlled internally for nonspecific binding of RBP1 to the 901 epitope. Interestingly, in both MDA-MB-231 and C8161.9, BRMS1 (Δ204–246) co-immunoprecipitated RBP1 more effectively (~1.5-fold) than full-length BRMS1 (Fig. 2, *A* and *B*).

BRMS1 Does Not Appear to Complex with Rb or p107 or to Modulate E2F-dependent Gene Expression—RBP1 binds Rb family members p105 (RB) and p107 (30–32). Rb proteins, in turn, bind E2F and tether RBP1 to E2F-responsive gene promoters. In this way, RBP1 directly suppresses transcription. We tested the hypothesis that BRMS1 is part of an RBP1·Rb·E2F complex; however, BRMS1 did not co-immunoprecipitate p105 or p107 in MDA-MB-231 (Fig. 2*A*) or C8161.9 cells (data not shown). Likewise, BRMS1 did not affect luciferase expression using an E2F-responsive promoter (data not shown). Taken together, these findings suggest that BRMS1 does not act as part of an RBP1·Rb·E2F complex and that BRMS1 might be part of a previously undescribed RBP1 complex that does not contain Rb.

BRMS1 Co-immunoprecipitates Several ^{35}S-labeled Proteins in MDA-MB-231—Anti-901 was used to co-immunoprecipitate BRMS1 from ^{35}S-labeled lysates from BRMS1-transfected MDA-MB-231. Vector-transfected cells were used as controls. In addition to BRMS1, several additional bands were evident, including prominent large proteins at ≥200 kDa, ~160 kDa, and ~65 kDa as well as less intense bands just below 50 kDa and another at ~30 kDa. (Fig. 3). Parallel experiments were performed using *BRMS1*-transfected C8161.9 and *Brms1* (murine ortholog; Ref. 7)-transfected 66cl4. Similar ^{35}S-labeled proteins were co-immunoprecipitated by anti-901 (data not shown). The pattern was reminiscent of previously published

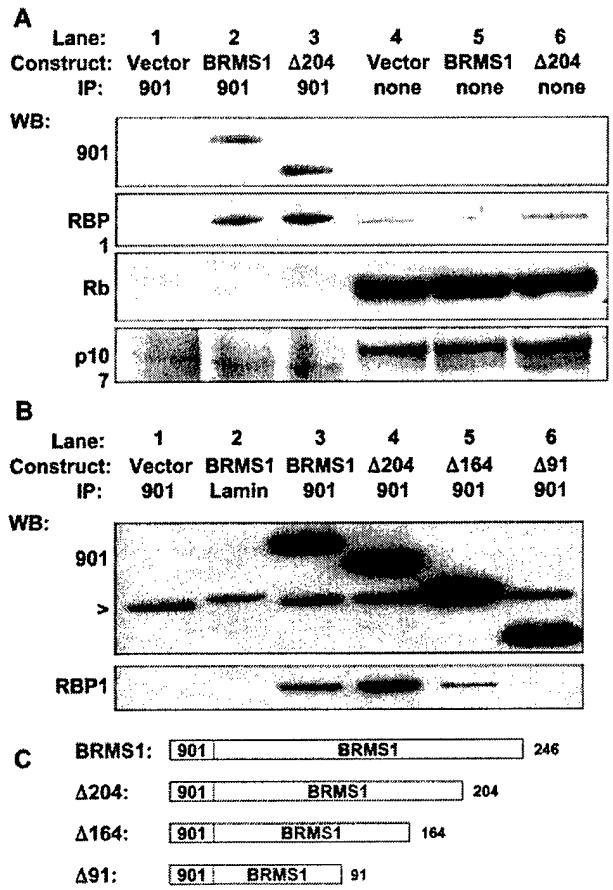

FIG. 2. **Binding to RBP1 is abrogated as C-terminal amino acids are removed from BRMS1, and BRMS1 does not co-immunoprecipitate Rb or p107.** *A*, whole cell lysates (1 mg) were prepared from MDA-MB-231 cells expressing 901-epitope-tagged BRMS1 or BRMS1(Δ204–246) (see *panel C*). BRMS1(Δ204–246) co-immunoprecipitated RBP1 (*lane 3*). Anti-901 did not pull down these proteins in vector-transfected cells (*lane 1*). To determine relative protein expression, 50 μg of protein lysate from each transfected construct was immunoblotted (*lanes 4–6*) (the exposure for α-901 shown here was not long enough to show expression in *lanes 4* and *5*). BRMS1 did not co-immunoprecipitate Rb or p107. *IP*, immunoprecipitation; *WB*, Western blot. *B*, whole cell lysates (1 mg) were prepared from C8161.9 cells expressing BRMS1 and BRMS1 deletion mutants (see *panel C*) with protein levels comparable with the clone expressing full-length BRMS1. The deletion mutants exhibited varying abilities to co-immunoprecipitate the above-mentioned proteins (*lanes 4–6*). Anti-901 (*lane 1*) and an irrelevant antibody (anti-Lamin A/C, *lane 2*) did not pull down RBP1 in vector-transfected cells. > indicates IgG light chain. *C*, schematic of BRMS1 deletion mutants. Equal loading of immunoprecipitate is inferred from the data, because equal intensity is observed by probing with anti-901.

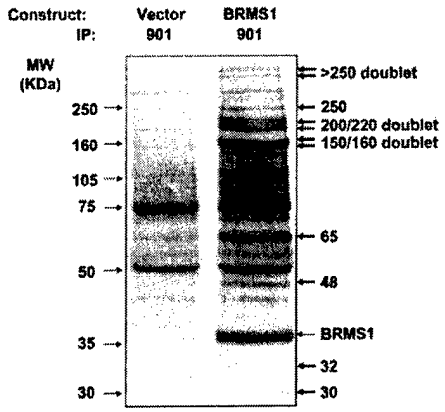

FIG. 3. **BRMS1 co-immunoprecipitated several proteins using ^{35}S-labeled whole cell lysates.** Using radiolabeled protein lysate from MDA-MB-231 cells, anti-901 was used to immunoprecipitate epitope-tagged BRMS1. Immunoprecipitation (*IP*) of BRMS1 revealed at least 12 co-immunoprecipitated proteins. *Arrows* with numbers indicate co-immunoprecipitated proteins and approximate molecular mass (*MW*) in kDa. > indicates IgG light chain.

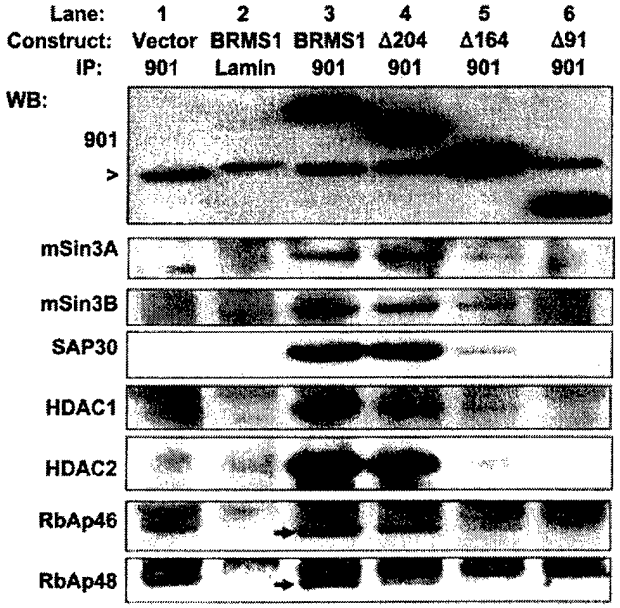

FIG. 4. **BRMS1 co-immunoprecipitated at least seven members of the mSin3 HDAC complex in C8161.9 human melanoma cells.** *A*, BRMS1 co-immunoprecipitated mSin3A, mSin3B, HDAC1, HDAC2, RbAp46, RbAp48, and SAP30 from whole cell lysates (1 mg) of stably transfected C8161.9 cells (*lane 3*). Whole cell lysates (1 mg) were also prepared from C8161.9 cells expressing BRMS1 deletion mutants (see Fig. 2*C*) with protein levels comparable with those of the clone expressing full-length BRMS1. Deletion mutants exhibited varying abilities to co-immunoprecipitate the above-mentioned proteins (*lanes 4–6*). Anti-901 did not pull down these proteins in vector-transfected cells (*lane 1*), and anti-Lamin A/C (an irrelevant antibody) did not pull down these proteins in BRMS1-transfected cells (*lane 2*). *IP*, immunoprecipitation; *WB*, Western blot.

results showing that RBP1 interacts with the mSin3·HDAC complex (31, 32). Specifically, HDAC1 and HDAC2 migrate at ~65/60 kDa. mSin3B and mSin3A migrate at ~160/150 kDa. These molecular mass proteins corresponded to the most prominent radiolabeled proteins co-immunoprecipitated with BRMS1 (Fig. 3). Therefore, we hypothesized that BRMS1 is a component of the mSin3·HDAC complex.

BRMS1 Is a Component of the mSin3·HDAC Complex in C8161.9 and MDA-MB-231—Immunoprecipitation of epitope-tagged BRMS1 followed by immunoblotting showed that BRMS1 pulled down seven proteins shown previously to be part of mSin3·HDAC complexes, namely mSin3A, mSin3B, HDAC1, HDAC2, SAP30, RbAp46, and RbAp48 (Fig. 4). The same proteins were not precipitated in vector-transfected cells (Fig. 4, *lane 1*), nor were they pulled down using an antibody to the nuclear protein Lamin A/C (Fig. 4, *lane 2*). Western blots demonstrated that BRMS1-associated proteins were present at comparable levels in both vector- and BRMS1-transfected cell lysates (data not shown), ruling out the possibility that vector-transfected cells had lower levels of mSin3·HDAC complex components. Interactions between BRMS1 and mSin3·HDAC were relatively strong, because they persisted in 0.5 M NaCl. Antibodies recognizing mSin3B, HDAC1, HDAC2, and SAP30 "reverse" co-immunoprecipitated BRMS1 in C8161.9 cells as well (Fig. 6*A*).

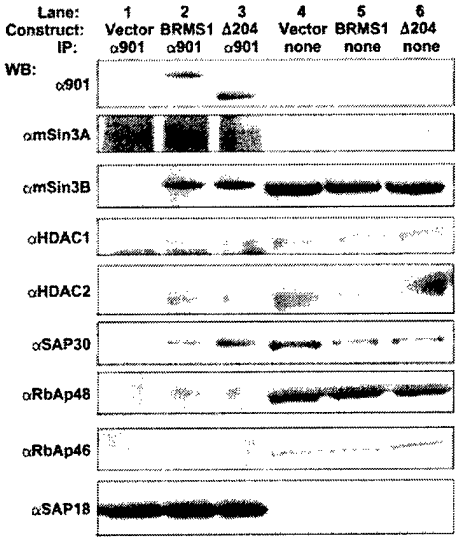

FIG. 5. **BRMS1 co-immunoprecipitated at least six members of a mSin3 histone deacetylase co-repressor complex in MDA-MB-231 human breast carcinoma cells.** *A*, BRMS1 co-immunoprecipitated mSin3A, mSin3B, HDAC1, HDAC2, RbAp48, and SAP30 from whole cell lysates (1 mg) of stably transfected MDA-MB-231 cells (*lane 2*). Whole cell lysates (1 mg) were also prepared from MDA-MB-231 cells expressing BRMS1 deletion mutant (Δ204–246) (see Fig. 2*C*) with protein levels comparable with the clone expressing full-length BRMS1. BRMS1(Δ204–246) also co-immunoprecipitated the above-mentioned proteins (*lane 3*). Anti-901 did not pull down these proteins in vector-transfected cells (*lane 1*). To determine relative protein expression, 50 μg of protein lysate from each transfected construct was immunoblotted (*lanes 4–6*) (the exposure for α-901 was not long enough to show expression in *lanes 4* and *5*). *IP*, immunoprecipitation; *WB*, Western blot.

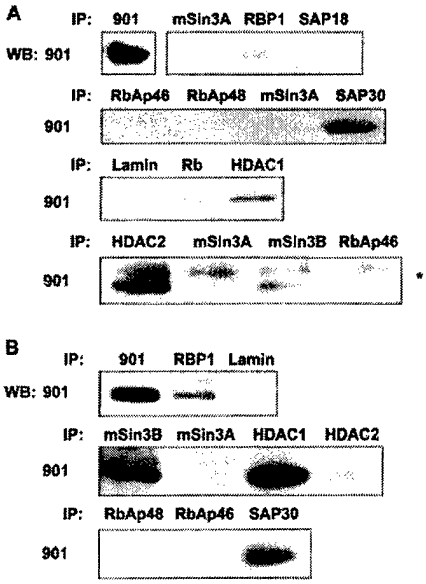

FIG. 6. **HDAC1, HDAC2, SAP30, RBP1, mSin3B, and mSin3A co-immunoprecipitated BRMS1.** *A*, in BRMS1-transfected C8161.9 cells, antibodies recognizing mSin3A, mSin3B, SAP30, HDAC1, HDAC2, and RBP1 co-immunoprecipitated BRMS1 from 1 mg of whole cell lysate. Antibodies directed against SAP18, RbAp46, RbAp48, and pRb did not co-immunoprecipitate BRMS1. Presence of the respective antigen recognized for co-immunoprecipitate was confirmed by re-staining the blots with the same antibody used for precipitation. *, in the *bottom panel*, increased exposure time was used to reveal co-immunoprecipitated BRMS1, causing a cross-reacting band of slower mobility to become visible. *B*, in BRMS1-transfected MDA-MB-231 cells, antibodies directed against mSin3B, SAP30, HDAC1, HDAC2, and RBP1 co-immunoprecipitated BRMS1 from 1 mg of whole cell lysate. Antibodies directed against mSin3A, SAP18, RbAp46, RbAp48, and pRb did not co-immunoprecipitate BRMS1. Anti-901 was used as a positive control. Presence of the precipitated antigen was verified by re-staining the blots with the antisera used for co-IP. *IP*, immunoprecipitation; *WB*, Western blot.

mSin3·HDAC complex proteins exhibited the same general interaction pattern with BRMS1 deletion mutants as did RBP1, with some exceptions. BRMS1(Δ204–246) co-immunoprecipitated mSin3A, mSin3B, SAP30, and HDAC2 at levels comparable with full-length BRMS1 (Fig. 4). However, BRMS1(Δ204–246) co-immunoprecipitated HDAC1, RbAp46, and RbAp48 less efficiently than full-length BRMS1 (reduced ~40% by densitometry) (Fig. 4). This discrepancy is evident on co-IP/immunoblots simultaneously probed for HDAC1 and mSin3B, clearly demonstrating differential binding (data not shown). BRMS1(Δ164–246) co-immunoprecipitated all mSin3·HDAC complex components significantly less efficiently than full-length BRMS1 (reduced ~90% by densitometry), whereas BRMS(Δ91–246) did not co-immunoprecipitate any complex proteins (Fig. 4).

To determine whether BRMS1 interacted with mSin3·HDAC complex proteins in human breast cancer cells, proteins were co-immunoprecipitated from BRMS1-transfected MDA-MB-231. Six mSin3·HDAC complex proteins, mSin3A, mSin3B, HDAC1, HDAC2, SAP30, and RbAp48 (Fig. 5), were pulled down with BRMS1. Co-IP in vector-transfected cells did not co-immunoprecipitate these proteins (Fig. 5, *lane 1*) despite the proteins being present in both vector- and BRMS1-transfected lysates (Fig. 5, *lanes 4* and *5*). As above, interactions persisted in 0.5 M NaCl. RbAp46, a member of the core mSin3·HDAC complex, did not co-immunoprecipitate with BRMS1 in MDA-MB-231 cells (Fig. 5). Antibodies recognizing mSin3B, SAP30, HDAC1, and HDAC2 co-immunoprecipitated BRMS1 in MDA-MB-231 (Fig. 6*B*). BRMS1(Δ204–246) co-immunoprecipitated mSin3·HDAC proteins at levels comparable with full-length BRMS1 (Fig. 5). In both melanoma and breast carcinoma cells, it was not possible to definitively demonstrate that BRMS1 co-immunoprecipitates SAP18, because SAP18 anti-sera also recognized a band at ~18 kDa in vector- and BRMS1-transfected cells (Fig. 5).

BRMS1 Interacts with a Subset of mSin3·HDAC Complexes—Many proteins that bind HDAC complexes are responsible for recruiting complexes to specific promoters. However, BRMS1 does not have a predicted DNA-binding motif, suggesting that it might serve a different role as a member of subsets of mSin3·HDAC complexes.

As a first step to evaluate those potential roles, the ability of BRMS1 to co-immunoprecipitate selected HDAC complex components was tested. Mad and Max were the first proteins shown to recruit the mSin3·HDAC to a specific promoter (33–35), but BRMS1 did not co-immunoprecipitate Mad1 or Max (data not shown). The unliganded nuclear hormone co-receptors SMRT and NCoR have also been reported to recruit the mSin3 (36–39), but there are contradictory data (40). In our system, BRMS1 did not co-immunoprecipitate SMRT or NCoR (data not shown). mSin3·HDAC interaction with MeCP2, a methyl CpG-binding protein, has also suggested that repression associated with DNA methylation may be mediated, in part, by deacetylation (41). Yet, BRMS1 did not co-immunoprecipitate MeCP2 (data not shown). Because the core HDAC subunit (HDAC1, HDAC2, RAp46, and RbAp48) is also present in the NuRD HDAC complex (16), we asked whether BRMS1 complexed with NuRD. BRMS1 did not co-immunoprecipitate Mi-2 or MTA1, two members of the NuRD complex (data not shown). HDAC3, which is related to HDAC1 and HDAC2 and can complex with RBP1 (32), did not co-immunoprecipitate with BRMS1 (data not shown).

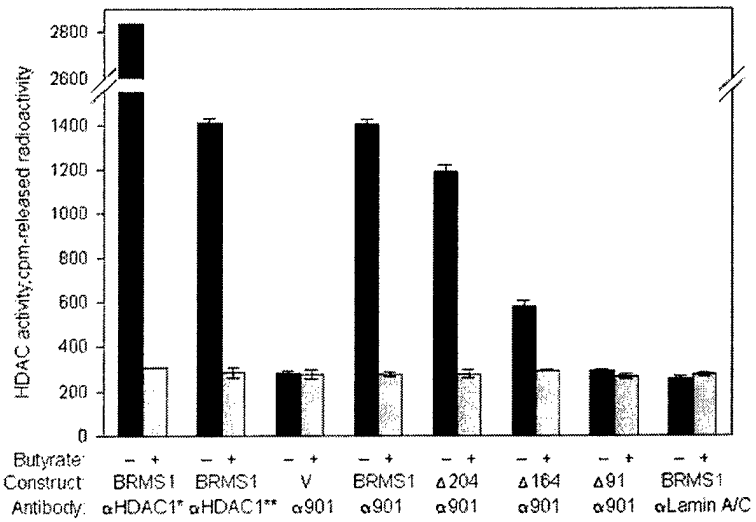

FIG. 7. **BRMS1 pulls down HDAC activity.** Whole cell lysate (6 mg of total protein) was prepared from BRMS1-transfected C8161.9 cells as well as from C8161.9 cells expressing BRMS1 deletion mutants (Δ204, Δ164, and Δ91) and vector-transfected (*V*) cells. Anti-901 was used to immunoprecipitate BRMS1 and BRMS1 deletion mutants from this lysate, and co-immunoprecipitated HDAC activity was measured. The HDAC inhibitor sodium butyrate (250 mM) was used to show that the release of ^{3}H-acetyl groups was due specifically to HDAC activity. Anti-HDAC1 was used as a positive control (*, 10 μg of anti-HDAC1 was used; **, 5 μg of anti-HDAC1 was used). Anti-Lamin A/C was used as a negative control. *Bars* with *error bars* represent mean ± S.E. for two independent experiments. See Fig. 2*C* for a schematic of the BRMS1 deletion mutants.

Taken together, these data suggest that BRMS1 exists in a specialized subset of mSin3·HDAC complexes rather than existing as an integral component of the complex. In other words, BRMS1 is not a ubiquitous member of mSin3·HDAC complexes.

BRMS1 Exists in Large (1.4 and 1.9 MDa) mSin3·HDAC Complexes as Well as Smaller Complexes Containing HDAC1—To determine the size of BRMS1·mSin3·HDAC complex(es) and the distribution of these molecules in complexes of various sizes, whole cell protein lysates from C8161.9 were subjected to Superose 6 size exclusion chromatography. Fractions were separated by PAGE, transferred to polyvinylidene difluoride, and immunoblotted for 901-BRMS1, HDAC1, SAP30, and mSin3B. These four proteins were chosen because they are core members of the complex. BRMS1 eluted in multiple peaks from the column with complex sizes ranging from ~100 to 2,000 kDa. BRMS1 elution was most prominent in peaks 5 and 6 (~1.7 MDa). HDAC1 also eluted in multiple peaks (fractions 4–22) with the majority present in fractions 8 and 9 (~ 1.4 MDa, Fig. 8*A*). SAP30 was detected in two peaks, one from fractions 4 through 14 and another from fractions 19 to 24, suggesting the existence of at least two complexes, the first >1 MDa and the second <200 kDa (Fig. 8*A*). mSin3B is detected uniformly in fractions 3–17, indicating involvement in complexes ranging from ~2 MDa to hundreds of kDa (Fig. 8*A*).

BRMS1 was immunoprecipitated from 420 μl of each fraction followed by PAGE and immunoblot. The vast majority (>90%) of BRMS1 was present in complexes ranging in size between 1.4 and 1.9 MDa (fractions 5–9 shown in *lanes 5–9*, Fig. 8*B*). BRMS1 also precipitated in fractions 10–23. HDAC1, SAP30, and mSin3B co-immunoprecipitated with BRMS1 in fractions 5–9, although SAP30 is most abundant in fractions 8 and 9 (*lanes 8* and *9*, Fig. 8*B*). HDAC1, however, also co-immunoprecipitated with BRMS1 in fractions 10–21, suggesting that BRMS1 can be involved in smaller complexes with HDAC1 (*lanes 10–21*, Fig. 8*B*).

BRMS1 Co-immunoprecipitates HDAC Activity—To determine whether BRMS1-associated HDAC1 and HDAC2 were enzymatically active, complexes were assessed for deacetylase activity in C8161.9. Full-length BRMS1 co-immunoprecipitated HDAC activity; BRMS1(Δ204–246) pulled down less

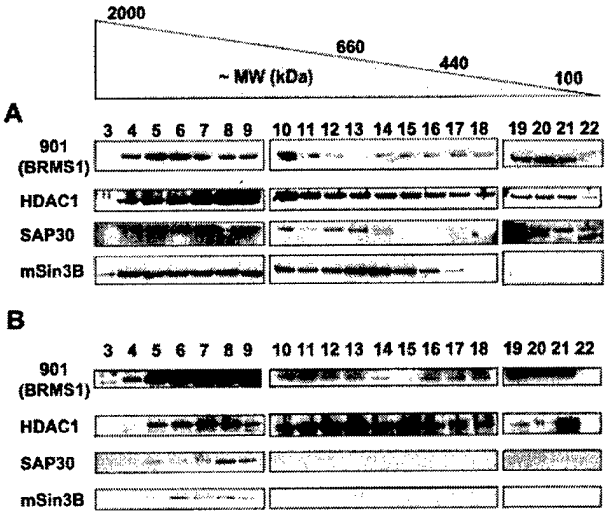

FIG. 8. **BRMS1 co-immunoprecipitated a large (~1.6 MDa) complex containing HDAC1, SAP30, and mSin3B as well as smaller complexes containing HDAC1.** *A*, elution profile of BRMS1, HDAC1, SAP30, and mSin3B in BRMS1-transfected C8161.9 cells. Whole cell lysate (3 mg total protein) was prepared and applied to a Superose 6 size exclusion column. Fractions (500 μl) were collected, and 20 μl of each fraction were subjected to SDS-PAGE and immunoblotting. *MW*, molecular mass. *B*, immunoprecipitation of BRMS1 within eluted fractions. Whole cell lysate (3 mg of total protein) was prepared from BRMS1-transfected C8161.9 cells and applied to a Superose 6 size exclusion column. Fractions (500 μl) were collected, and anti-901 was used to immunoprecipitate BRMS1 from 420 μl of each fraction. Immunoprecipitated complexes were subjected to PAGE and immunoblotting.

HDAC activity. BRMS1(Δ164–246) co-immunoprecipitates still less HDAC activity, whereas BRMS1(Δ91–246) pulled down only background activity (Fig. 7). This pattern is reminiscent of the pattern of interaction with HDAC1 seen by immunoblot (Fig. 4). As a positive control, anti-HDAC1 antibodies were able to pull down HDAC activity (Fig. 7) proportionate to the amount of antibody used (*i.e.* when 2× anti-HDAC1 was used, double the HDAC activity was precipitated). These results show that only a small portion of the HDAC1

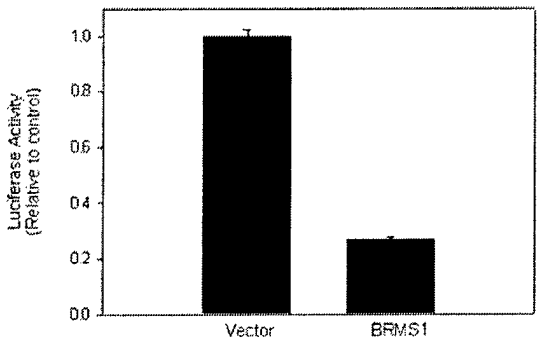

FIG. 9. **BRMS1 represses transcriptional activity *in vivo*.** Using a luciferase reporter assay containing four GAL4 binding sites upstream of the myelomonocytic growth factor minimal promoter, BRMS1 strongly repressed (~80%) basal transcription compared with the pBIND vector alone.

activity present in the protein lysate is being measured. Vector-transfected cells and co-IP with anti-Lamin A/C served as negative controls (Fig. 7).

BRMS1 Represses Transcription in Luciferase Reporter Assays—On the basis of its physical interactions with mSin3 and HDAC1, it was predicted that BRMS1 would repress transcription. To investigate this prediction, we measured the effect of BRMS1 on transcription using a luciferase reporter containing four GAL4 binding sites upstream of the myelomonocytic growth factor minimal promoter. BRMS1 strongly repressed (~80%) basal transcription compared with the pBIND vector alone (Fig. 9).

DISCUSSION

Epigenetic regulation of the metastatic phenotype was proposed in 1889 when Sir Stephen Paget recognized that tumor cells colonize certain organs preferentially based, in part, upon how they respond to signals from the microenvironment (42). Trainer and co-workers later showed that treatment of murine melanoma cells with the DNA de-methylating agent 5-azacytidine resulted in reversible reduction of metastatic lung colonization (43). Recent studies have shown that treatment of cells with 5-azacytidine can induce expression of the metastasis suppressor genes *Nm23* (44) and *KAI1* (45). Links between metastasis and HDAC activity first became apparent when the breast cancer metastasis promoting gene, *MTA1*, was identified as a component of the NuRD·HDAC complex (46, 47). *MTA1* has subsequently been shown to repress estrogen receptor-dependent transcription in an HDAC-dependent manner (48). Likewise, loss of expression of heterochromatin protein 1 (HP1) has been associated with acquisition of metastatic potential in human breast cancer (49). Together, these findings support the hypothesis that regulation of the transcriptome by a variety of mechanisms is a critical determinant of cancer spread. The findings reported here represent the first direct evidence that a metastasis suppressor gene is a component of an HDAC complex. It is possible that specialized HDAC complexes may promote (as implied by MTA1) or inhibit (as implied by BRMS1) cancer metastasis. The data compel the hypothesis that metastasis is regulated, at least in part, by histone deacetylase activity, chromatin remodeling, and/or transcriptional repression.

Connections between HDAC activity and cancer have emerged in recent years, stemming from observations that HDAC inhibitors, such as trichostatin A and suberoylanilide hydroxamic acid (SAHA), can induce growth arrest, differentiation, and/or apoptosis in transformed cultured cells (50). In pre-clinical animal models, HDAC inhibitors have demonstrated impressive anti-tumor activity which, in turn, led to several ongoing HDAC inhibitor clinical trials (50–53). The data presented here, along with data regarding MTA1 and HP1 cited above, are consistent with the hypothesis that HDAC inhibitors may influence not only primary tumors but also distant metastases.

Interestingly, BRMS1 appears to be part of a protein family in which all of the characterized members are components of the mSin3·HDAC complex. During the original yeast two-hybrid screen, two cDNA clones identified as FLJ00052 were identified in the prostate library. As studies were underway to follow-up RBP1, mSin3, and HDAC findings, FLJ00052 was re-designated by GenBank™ as mSds3, the mammalian ortholog of *Saccharomyces cerevisiae* Sds3. Sds3 has been implicated in gene silencing through a Sin3·Rpd3 pathway (Rpd3 in a yeast HDAC1 ortholog) and is an integral component of the yeast Sin3·Rpd3 complex that is required for histone deacetylase activity (17, 54). BRMS1 shares 18% identity and 49% similarity with a large region of yeast Sds3 and 23% identity and 49% similarity with mSds3. mSds3, analogous to its yeast ortholog, is a component of the mSin3·HDAC complex, stabilizes HDAC1 within the complex, and augments HDAC activity (17). Another predicted mammalian protein of unknown function (designated MGC11296) is homologous to both Sds3 and BRMS1. Homology to BRMS1 is particularly strong (58% identity; 79% similarity for the C-terminal 196 amino acids of BRMS1 and the N-terminal 196 amino acids of MGC11296). The high level of sequence similarity between these molecules, combined with their associations with mSin3·HDAC complexes, suggests the existence of a BRMS1 family of proteins that may play a crucial role in altering metastasis by regulating the so-called histone code (29, 49).

Although specific role(s) for BRMS1 within mSin3·HDAC complexes remain to be elucidated, the following lines of evidence suggest that the metastasis suppressor may be involved in recruiting and stabilizing HDAC1 and/or modulating HDAC activity. 1) BRMS1 forms small complexes (~100 kDa and greater) with HDAC1 but forms only large complexes (~1.4 to 1.9 MDa) with Sin3B and SAP30 (Fig. 8*B*). 2) BRMS1 has distinct binding site(s) for the HDAC1·RbAp46/48 core subunit as compared with the rest of the complex (mSin3A, mSin3B, SAP30, HDAC2, and RBP1) as demonstrated by BRMS1-(Δ204–246) binding less effectively to HDAC1·RpAp46/48 than does full-length BRMS1; in contrast, BRMS1(Δ204–246) binds the remaining complex components as effectively (Fig. 4). 3) The C-terminal 42 amino acids of BRMS1 appear to stabilize HDAC1·RbAp46/48 within the complex, as deletion of these residues specifically compromises binding to these three components (Fig. 4). 4) Both characterized BRMS1 family members (Sds3 and mSds3) are required for optimal HDAC activity, and mSds3 specifically stabilizes HDAC1 within the mSin3 complex.

Although remarkably similar in breast carcinoma and melanoma cell lines, BRMS1·mSin3·HDAC complexes were distinct. RbAp46 complexes with BRMS1 were not detected in MDA-MB-231 (Fig. 5), and the interaction with RbAp48 appeared less robust than in C8161.9 (compare Figs. 4 and 5). Differential binding of BRMS1(Δ204–246) to the HDAC1-RbAp46/48 subunit in C8161.9 was not observed in MDA-MB-231 (compare Figs. 4 to 5). At this juncture, it is not possible to distinguish whether the differences are due to cell origin or presence of mutations that abrogate interactions of RbAp46 with BRMS1·mSin3a. BRMS1-transfected MDA-MB-231 cells are suppressed for metastasis less than C8161.9 (40–90 *versus* 90–100%). It is tempting to speculate that differences in metastasis suppression may be related to a differential interaction between BRMS1 and the HDAC1-RbAp46/48 subunit.

Preliminary data obtained with the BRMS1 deletion mu-

tants reported here are consistent with a correlation between complexes involving BRMS1, mSin3, and HDAC and metastasis suppression. C8161.9.BRMS1(Δ164–246) and (Δ91–246) clones (mSin3 interactions severely impaired or lost; Fig. 4) fail to suppress metastasis (data not shown). However, more refined BRMS1 mutants will be required to determine whether binding to the mSin3·HDAC complex is necessary for metastasis suppression. Systematic site-directed mutagenesis of BRMS1 coupled with metastasis assays are underway.

In summary, the metastasis suppressor BRMS1 is shown here to interact with enzymatically active mSin3·HDAC complexes. BRMS1 is also shown to form smaller complexes with HDAC1 and to repress transcription when recruited to a promoter region. Besides defining a milieu in which BRMS1 works within cells, the data presented here imply that specific downstream mediators, regulated in part by HDAC activity, are critical to controlling metastatic behavior. Indeed, preliminary cDNA microarray and proteomic studies have identified a limited number of BRMS1-regulated genes.[2] Understanding the role(s) of BRMS1·mSin3·HDAC complexes in the regulation of gene expression promises to provide insights into metastasis suppression, HDAC-mediated chromatin regulation, and BRMS1 physiology in noncancerous cells.

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Commentary

Microarrays bring new insights into understanding of breast cancer metastasis to bone

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Abstract

Using a microarray approach, Kang and colleagues identified several genes involved in the generation of breast cancer metastasis in bone and demonstrated their roles in bone colonization *in vivo*. Their findings and interpretations are reviewed in the context of recent array studies that compared gene expression in primary tumors and metastases. RNA expression array results have already demonstrated value in predicting whether metastases will develop in patients. They have also shown that expression patterns are similar in primary tumors and metastases. The latter data have invited re-examination of long-held notions related to mechanisms of metastasis. While the arrays show promise for improving diagnostic capability in breast cancers, ascribing mechanistic interpretations to correlative data should be done with extreme caution. Kang and colleagues' paper in *Cancer Cell* elegantly reinforces the concepts that efficiency of the metastatic process is dependent on the coordinated expression of multiple genes and that the expression of metastasis-associated genes is sometimes dependent on the microenvironment in which cells find themselves.

Keywords: bone metastasis, metastatic inefficiency, metastasis genes, microarray, organotropism

Introduction

Kang and colleagues, in a recent issue of *Cancer Cell*, identified genes that promote breast carcinoma metastasis to bone [1]. Transcriptomes were compared between the parental MDA-MB-231 human breast carcinoma cell line and a variant selected one time *in vivo* for bone colonization from MDA-MB-231 (231-bone). They identified 43 overexpressed genes and 59 underexpressed genes. This pattern is referred to as a 'bone metastasis signature'. Among the overexpressed genes were matrix metalloproteinase 1, IL-11, a chemokine receptor for SDF-1 (CXCR4) and connective tissue-derived growth factor. Cotransfection of gene combinations into the parental MDA-MB-231 human breast carcinoma cell line resulted in populations as efficient at bone colonization as 231-bone, whereas transfection of individual cDNAs modestly increased the bone metastatic efficiency. Additionally, and importantly, Kang and colleagues demonstrated that bone colonizing clones pre-existed within the parental populations by single cell cloning.

Their results provide insights into the underlying mechanisms of cancer metastasis as well as support for Paget's 'seed and soil' hypothesis [2] regarding organotropism of metastases at molecular and functional levels. The findings of Kang and colleagues also contribute to the resolution of recent discussions regarding whether metastasis competent cells are rare variants or are prevalent within primary tumors. Furthermore, the findings support assertions that there are genes that specifically contribute to metastasis.

Microarrays as molecular pathology tools

Expression microarrays have the potential to revolutionize the practice of pathology by providing a molecular 'signature' that is characteristic of the cancer subtype [3,4].

231-bone = variant selected one time *in vivo* for bone colonization from MDA-MB-231; IL = interleukin.

Van't Veer and colleagues used a 70-gene set to identify and define a 'poor prognosis' transcriptome in breast cancer [5], which was subsequently used to predict the likelihood of metastasis development and patient survival [6]. One can envision a scenario whereby pharmacogenomic assays will stratify patients needing aggressive treatment (i.e. metastasis predisposed) versus less aggressive treatment (i.e. unlikely to develop microscopic metastases) [7]. Results from the *Cancer Cell* article further imply that arrays might further predict where metastases will develop. If so, therapy could be targeted to sites where metastases are possible, rather than simply administering toxins systemically.

Metastatic cells in the primary tumor: rare or predominant?

Microarray data have also been used to challenge long-held notions that metastases arise from a rare subset of cells within a primary tumor [5,6,8,9]. It is important, however, to consider the methodologies used and the interpretations that arise from the findings represented. Ramaswamy and colleagues, who compared primary tumors and metastases from multiple tumor types, found the array patterns to be nearly identical [8], leading them and other workers [5,9–11] to infer that metastatic potential is hardwired into tumor cells. As with the data of Van't Veer and colleagues, the microarrays were performed using bulk measurements (i.e. samples contained mixtures of RNA from multiple tumor cells). The samples were also 'contaminated' with normal stromal cells.

The conclusions, that a predominance of neoplastic cells had already acquired metastatic potential and that metastases arose from early oncogenic changes rather than specific events that control metastasis [9], can be challenged on the basis of other data. In short, while microarray differences are predictive of patient outcome, they neither address the issue of metastasis-competent cell prevalence [12,13] nor do they preclude the existence of metastasis-controlling genes. Likewise, microarray data cannot distinguish contributions from noncancer cells since the starting materials were not purely neoplastic cells.

Most tumors are clonal in origin, yet are heterogeneous for multiple phenotypes at diagnosis. Generation of heterogeneity is the result of genetic instability that, in turn, leads to variants with differences in metastatic potential, as demonstrated by selection of metastatic subpopulations within a tumor [12]. If one invokes the principles of Luria and Delbrück [14], the prevalence of metastatic cells within a tumor would depend on the time at which the metastatic cells emerged (i.e. earlier in progression would yield a higher proportion). While reasonable, this simple notion is complicated by the multistep nature of metastasis. Finely choreographed expression (increased or decreased) of multiple genes is required for metastatic competence. In addition, expression changes in tumor cells are superimposed by tumor cell interactions with the host microenvironment at virtually every step. If a cell is (rendered) incapable of completing any step, it is non-metastatic. In other words, every step of the metastatic cascade is rate limiting. As a result, it is not surprising that metastasis is highly inefficient [15]. Metastatic inefficiency is a critical parameter with regard to interpreting the microarray results for mechanistic insights.

Butler and Gullino, for example, showed that $(1–4) \times 10^6$ cells/g tumor per day are shed into the vasculature [16]. Shedding of cells from a primary tumor is only one step of the metastatic cascade, and the data from Butler and Gullino would argue that a substantial fraction of neoplastic cells have this ability. The ability of disseminated cells to complete subsequent steps in the metastatic cascade is not inherent *a priori*, however, as would be inferred by the relative infrequency of overt metastases. The issue of colonization is not addressed by any of the microarray data. It is known that most metastases are clonal in origin [17]; addressing the issue of metastasis genes therefore requires direct comparison of primary cells and multiple metastases. It is probable that many tumors contain subpopulations that have accumulated some, but not all, of a metastasis signature. When a tumor has a sufficient proportion of cells that express at least one of the 'poor prognosis' genes, it follows that it has increased likelihood for cells coexpressing the entire complement of metastasis-associated genes. Unfortunately, bulk array data (i.e. cells are not microdissected) cannot discern whether the appropriate pattern of expression exists within individual cells versus within the entire population.

The data from Kang and colleagues illustrate this argument elegantly. Several single cell clones showed differential expression of one or more of the bone metastasis profile genes; however, only a small fraction showed coordinate expression. Likewise, transfection of osteopontin or IL-11 alone resulted in only a modest increase in bone colonization efficiency. However, cotransfection of these genes (and others described earlier) resulted in higher metastatic efficiency. In other words, osteopontin and IL-11 were not sufficient for bone colonization. Of course, interpretation must be done with caution since there was already a baseline of 30% colonization in bone (i.e. what other genes were 'on' or 'off' already?).

This issue raises an important point that limits all experimental metastasis studies with current technology and reagents; virtually all human breast cancer cell lines were derived from metastases. Baseline measurements will be skewed as a result, but the magnitude of bias is not known. In the context of bone metastasis, it is likewise important to note that none of the commonly used human breast carcinoma cell lines were derived from bone metas-

tases (i.e. most, including MDA-MB-231, were isolated from pleural effusions). Finally, interpretation is always complicated by the artificial nature of intracardiac injection models since none of the current human breast carcinoma cell lines colonize bone from an orthotopic site. In short, xenograft models of bone metastases have serious limitations. Such issues are not unique to Kang and colleagues' article; however, they are endemic to the field. It is important to emphasize that such complications do not undermine their experiments, but point out the need for caution when interpreting the data.

Metastasis genes that control colonization

In Kang and colleagues' article, bone colonization was affected in the transfectants while adrenal colonization was not impacted by overexpression of these genes. This implies that metastasis to each organ will be characterized by different expression signatures. As a result, the concept posited earlier regarding organotropism expression signatures can be added to the hierarchy of array analysis.

The most common site of breast cancer metastasis is to the bone. This observation was the crux of Paget's seed and soil hypothesis. In short, tumor cells (the seed) must respond favorably to the tissue microenvironment (the soil) in order to form overt metastases. At the core of Paget's hypothesis is the interaction between the tumor and the host. This notion is nicely presented by Hunter and colleagues, who showed that a single oncogenic event could lead to differential metastasis, depending upon the host background [18,19].

Kang and colleagues showed that CXCR4, a chemokine receptor for the SDF-1 ligand highly expressed in bone, is more highly expressed in the 231-bone variant. Their findings are consistent with clinical data in breast cancer [20]. The prevailing hypothesis is that tumor cells respond to SDF-1 chemoattractant gradients to preferentially migrate to bone. If this hypothesis were true, then bone overexpressing SDF-1 would be more commonly colonized than bone with low expression. To the best of my knowledge, this hypothesis has not been directly tested. Nonetheless, the cumulative data emphasize that DNA expression is dependent, to some extent, upon exogenous signals. Interestingly, many of the genes expressed in 231-bone are responsive to transforming growth factor beta, which is prevalent in bone.

The conclusion that metastatic potential is determined by early oncogenic events and not metastasis-specific genes [5,6,8,9] is inconsistent with a growing literature demonstrating the existence of genes that suppress metastasis but that have no effect on tumorigenicity (reviewed in [21,22]). Studies on metastasis-promoting genes, which are the preferred targets for diagnostic studies, are complicated by the requirement for coordinated expression of multiple genes. As a result, false-negative results are more likely when determining the function of metastasis-promoting genes than the function of suppressors (since blocking any step inhibits metastasis) [23]. Studies designed to explore promoting genes therefore generally start with a baseline of metastasis and look for an increase. While entirely appropriate based upon experimental considerations, interpretation of experiments is not as straightforward as that for suppression.

Additionally, data are accumulating that some metastasis suppressors may exert organ-specific effects (i.e. growth of tumor cells is site specific). Goldberg and colleagues showed, for example, that metastasis-suppressed melanoma variants grow in the skin and disseminate to the lung. Once in the lung, however, they remain quiescent for extended periods [24]. Similarly, the Rinker-Schaeffer laboratory has shown that the metastasis suppressor MKK4 exerts a similar growth suppression at the secondary site while not affecting primary tumor growth [25,26]. In both these studies, tumor cells complete every step of the metastatic cascade except colonization. Both of these examples support findings from the Chambers and Groom laboratories showing high-frequency dissemination and extravasation, but showing low-frequency proliferation at the secondary site [27,28]. The cumulative data again emphasize the necessity for coordinated expression of genes to complete the entire metastatic process. It remains unclear at this time whether the coordination of gene expression is contemporaneous or sequential, synergistic or additive, or even whether metastatic competence is determined by unique or complementary pathways.

An unmistakable conclusion from the presented examples is that metastatic cells respond to environmental signals differently to nonmetastatic cells. It is thus easy to extrapolate that epigenetic regulation of some genes is crucial to metastasis control. The only way to address these complex issues will be to directly compare transcriptomes and proteomes in matched primary tumors and metastases (preferably from multiple sites) microdissected from adjacent normal tissues. Even then, until reliable and reproducible methods for single cell analyses are at hand, there will remain questions regarding interpretation.

In summary, the data by Kang and colleagues beautifully highlight the multigenic nature of cancer metastasis and show that some, but not all, cells in a primary tumor express the entire cadre of genes necessary to colonize bone. Their data, combined with that from many other laboratories, supports the notion that specialized subpopulations within the primary tumor can complete the metastatic process. Their data also highlight the importance of arrays for predicting clinical outcome, but emphasize the need for caution when ascribing a mechanism.

Competing interests

None declared.

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A small molecule antagonist of the $\alpha_v\beta_3$ integrin suppresses MDA-MB-435 skeletal metastasis

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Key words: breast cancer, metastasis, bone, MDA-MB-435, green fluorescent protein

Abstract

Introduction: Breast cancer is one of the most common malignancies affecting women in the United States and Europe. Approximately three out of every four women with breast cancer develop metastases in bone which, in turn, diminishes quality of life. The $\alpha_v\beta_3$ integrin has previously been implicated in multiple aspects of tumor progression, metastasis and osteoclast bone resorption. Therefore, we hypothesized that the $\alpha_v\beta_3$-selective inhibitor, S247, would decrease the development of osteolytic breast cancer metastases. *Materials and methods*: Cells were treated *in vitro* with S247 and assessed for viability and adhesion to matrix components. Athymic mice received intracardiac (left ventricle) injections of human MDA-MB-435 breast carcinoma cells expressing enhanced green-fluorescent protein. Mice were treated with vehicle (saline) or S247 (1, 10, or 100 mg/kg/d) using osmotic pumps beginning either one week before or one week after tumor cell inoculation. Bones were removed and examined by fluorescence microscopy and histology. The location and size of metastases were recorded. *Results and conclusions*: IC_{50} for S247 adhesion to $\alpha_v\beta_3$ or $\alpha_{IIB}\beta_{3a}$ substrates was 0.2 nM vs. 244 nM, respectively. Likewise, S247 was not toxic at doses up to 1000 μM. However, osteoclast cultures treated with S247 exhibited marked morphological changes and impaired formation of the actin sealing zone. When S247 was administered prior to tumor cells, there was a significant, dose-dependent reduction (25–50% of vehicle-only-treated mice; $P = 0.002$) in osseous metastasis. Mice receiving S247 after tumor cell inoculation also developed fewer bone metastases, but the difference was not statistically significant. These data suggest that, in the MDA-MB-435 model, the $\alpha_v\beta_3$ integrin plays an important role in early events (e.g., arrest of tumor cells) in bone metastasis. Furthermore, the data suggest that $\alpha_v\beta_3$ inhibitors may be useful in the treatment and/or prevention of breast cancer metastases in bone.

Abbreviations: CMF-DPBS – calcium- and magnesium-free Dulbecco's phosphate-buffered saline solution; FACS – fluorescence-activated cell sorting; GFP – enhanced green-fluorescent protein; HEPES – hydroxyethylpiperazine ethane sulfonic acid

Introduction

Breast cancer frequently spreads to bone, typically resulting in osteolysis which, in turn, leads to severe pain, fracture, disruption of calcium homeostasis and a poor quality of life. Osteolysis appears to be due primarily to tumor cells disrupting the normal bone forming unit by tilting the balance toward osteoclast activity (reviewed in [1, 2]). Recent use of bisphosphonates has had some palliative effects by decreasing bone resorption [3–5]; however, lost bone is typically not replaced [6]. These observations suggest that there remains much that is still not known regarding the interactions between tumor cells and the bone microenvironment.

One reason for the poor understanding is that osteolysis is a relatively late event in the pathogenesis of bone

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metastasis. Prior to proliferation and induction of osteolysis, metastatic cells must first invade from the primary tumor and detach, enter the vasculature, move to and be detained in the bone vascular sinusoids, then migrate into the regions rich in trabecular bone [7]. How tumor cells interact at secondary sites determines the efficiency of metastasis and governs organotropism. Recent evidence suggests that tumor cells respond to chemokines, chemotactic factors that recruit the cells to bone [8, 9]. Tumor cells then use a variety of adhesion molecules to adhere to endothelial cells and extracellular matrices [10]. In addition, adhesion at the secondary site controls, in part, anti-apoptotic [11] and proliferative signals [12, 13].

The integrins are a family of heterodimeric transmembrane glycoproteins, each comprised of non-covalently linked α and β subunits [14]. Integrins are involved in multiple cell–cell and cell–matrix interactions, including adhesion, migration and response to soluble ligands – all integral steps to the metastatic cascade [15].

The $\alpha_v\beta_3$ heterodimer has been associated with both breast cancer [16, 17] and osteoclast function [18–22], suggesting that it may contribute to osteotropism of breast cancer as well as carcinoma-induced osteolysis. It is highly expressed in normal breast epithelium and heterogeneously expressed among invasive breast carcinomas; but, $\beta_v\alpha_3$ is over-expressed in metastatic breast cancer cells within bone [23]. As an integrin, $\alpha_v\beta_3$ may mediate adhesion and migration in early steps of bone colonization, such as arrest, adherence or extravasation [24–26] and has a particularly high affinity for bone-associated matrix proteins such as osteopontin and bone sialoprotein [27]. $\alpha_v\beta_3$ also binds vitronectin, fibronectin, fibrinogen, laminin and collagen [28]. However, the integrin has also been linked to later steps, including tumor growth, angiogenesis [29, 30] and osteolysis [19]. Integrin $\alpha_v\beta_3$ can associate with platelet-derived growth factor (PDGF) receptor, insulin receptor, and vascular endothelial growth factor receptor 2 (VEGF-R2) [31–34] and may play a key role in regulating tumor cell survival and proliferation [14, 35]. Indeed, expression of activated $\alpha_v\beta_3$ integrin has recently been associated directly with the metastatic potential of MDA-MB-435 breast carcinoma [36, 37].

In addition to roles it plays in metastatic tumor cells, integrin $\alpha_v\beta_3$ also operates in several capacities within bone. It is the predominant integrin expressed on osteoclasts and may function in osteoclast differentiation, syncytium formation, sealing zone adhesion and migration [25]. Essential to polarization of osteoclasts and their interaction with bone matrix, $\alpha_v\beta_3$ localizes to both the ruffled border and sealing zone of osteoclasts [19, 38, 39]. Osteoclast motility and resorptive polarization is carefully regulated by mechanisms including changes in the conformation of $\alpha_v\beta_3$ [39]. Horton et al. showed that arg-gly-asp (RGD) peptides and anti-vitronectin antibody (23C6) inhibited dentine resorption and cell spreading by osteoclasts. $\alpha_v\beta_3$ is also the primary integrin receptor of osteopontin (OPN), abundant in bone, involved in $\alpha_v\beta_3$-mediated osteoclast adhesion and haptotaxis [18]. Interestingly, osteopontin is produced by metastatic breast cancer cells and expression often correlates with metastatic proficiency [40–42].

Given the evidence for roles of $\alpha_v\beta_3$ in both metastasis and osteolysis, we hypothesized that $\alpha_v\beta_3$ might be an important mediator of breast cancer metastasis to bone. As a corollary, we predicted that antagonists of $\alpha_v\beta_3$ would inhibit metastasis to bone and/or decrease osteolysis. To test this hypothesis, we used a peptidomimetic inhibitor of $\alpha_v\beta_3$, S247. This and related small molecules have been shown to significantly decrease *in vitro* bone resorption [20, 43] and inhibit *in vivo* oophorectomy-induced osteoporosis [44]. It is also a potent antagonist in cell-based assays including adhesion of human $\alpha_v\beta_3$-transfected HEK-293 cells on vitronectin, and osteoclast adhesion and actin ring formation *in vitro*. Furthermore, it has also been demonstrated that S247 decreases liver metastasis of CT26 colon cancer cells following splenic injection by impairing angiogenesis in metastases [45]. Likewise, lung colonization from orthotopically injected human tumor xenografts was inhibited significantly by S247 (> 90%, [46]). We show that S247 is a potent ($\alpha_{IIb}\beta_3$-selective) antagonist of, $\alpha_v\beta_3$-mediated cell adhesion on vitronectin and inhibits osteoclast adhesion and actin ring formation *in vitro*. We also demonstrate that S247 significantly inhibits formation of osteolytic breast cancer metastasis to bone.

Materials and methods

Cell lines and culture

Metastatic human breast carcinoma cell line, MDA-MB-435 (435) was a generous gift from Dr Janet E. Price (University of Texas–M.D. Anderson Cancer Center, Houston, Texas) and was stably transfected with pEGFP-N1 (BD Biosciences Clontech, Palo Alto, California) as previously described [47]. Cells were maintained in a 1:1 mixture of Dulbecco's-modified Eagle's medium and Ham's F12 medium (DMEM/F12; Invitrogen, San Diego, California), supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.02 mM non-essential amino acids and 5% fetal bovine serum (Atlanta Biologicals, Norcross, Georgia). Neomycin-resistant lines were maintained in 500 μg/ml geneticin (G418; Invitrogen). All cultures were confirmed negative for *Mycoplasma spp.* infection using a PCR-based test (TaKaRa, Shiga, Japan).

Recently, microarray data have observed melanoma gene expression in MDA-MB-435 cells which has led some to conclude that MDA-MB-435 cells are of melanocyte origin, not breast [48, 49]. However, the MDA-MB-435 cells also express and secrete milk lipids, which are breast epithelium-specific markers [50]. Combined with the clinical data showing that the patient from whom the MDA-MB-435 cell line was derived had breast cancer [51, 52] and no evidence of or history of melanoma, the most logical conclusion is that MDA-MB-435 is a breast epithelial cell lines which has dedifferentiated or undergone lineage infidelity. Importantly, melanomas also colonize bone.

293/$\alpha_v\beta_3$ cells were created by stable transfection of the human embryonic kidney 293 cell line with cloned full-length human cDNA encoding the integrin beta-3 subunit. High surface expression of $\alpha_v\beta_3$ integrin complex was confirmed by flow cytometry using readily available commercial antibodies.

Integrin function assays

To measure the effects of compounds on $\alpha_v\beta_3$ function, 96-well plates were coated at 4 °C overnight with a solution of 0.1 μg/ml purified vitronectin in TS buffer (25 mM Tris, pH 7.4; 150 mM NaCl), and then blocked with 200 μl of TS/1% bovine serum albumin (BSA) for 1 h at 37 °C. Freshly resuspended 293/$\alpha_v\beta_3$ cells were incubated for 30 min at 37 °C in the presence of the indicated doses of compounds in Hank's-buffered balanced salt solution (Invitrogen) supplemented with 0.1% BSA, 25 mM HEPES, 200 μM $MnCl_2$. Cells (1×10^5) from each sample were added to triplicate wells of the ligand-coated plate, and adhesion was allowed to occur for 30 min at 37 °C. After washing thrice with adhesion buffer, 100 μl of the buffer was added per well, followed by 100 μl of 0.6 mg/ml p-nitrophenyl-phosphate (Sigma), 50 nM sodium acetate, 0.5% Triton-X-100, pH 5. Color was allowed to develop for 30 to 45 min at 37 °C before the reaction was terminated with 50 μl NaOH (1N). Absorbance was measured at 405 nm and IC_{50} values were determined by fitting a four-parameter logistic, non-linear model using a Microsoft Excel spreadsheet macro program.

The effects of S247 on integrin $\alpha_{IIb}\beta_3$ activity were characterized by a solid phase competitor displacement assay using purified integrin as previously described [44].

F-actin ring formation

Human bone marrow derived $CD34^+$ cells were cultured with RANKL and M-CSF for approximately 14 days to induce osteoclast differentiation. Osteoclast-like cells were detached from culture dishes using a non-enzymatic cell dissociation buffer (Gibco BRL, Rockville, Maryland), then transferred into 24-well cell culture plates containing 15-mm diameter borosilicate glass coverslips (Bioworld, Dublin, Ohio) that were previously coated overnight with 100% FBS to mimic the bone surface. Cells were allowed to adhere to the coverslips for 20 h, after which testing agents (e.g., S247) were added and incubated for 4 h. Cells were treated with RANKL for the entire culture period. They were fixed with a 4% (v/v) paraformaldehyde solution for 30 min, and permeabilized with a solution containing 20 mM hydroxy-ethyl-piperazine-ethanesulfonic acid (HEPES), 300 mM sucrose, 50 mM NaCl, 3 mM MgCl2 and 0.5% Triton X-100, on ice for 30 min. Fixed cells were blocked with 0.5% BSA in PBS for 30 min, stained with phalloidin-Texas Red for 1 h, and 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) for 10 min to visualize F-actin rings and nuclei, respectively. The number of osteoclast-like cells containing clearly delineated actin rings was determined by counting by fluorescence microscopy.

S247 treatment

Alzet® mini-osmotic pumps (Durect, Cupertino, California) were implanted subcutaneously on the dorsal trunk of treated mice. Pumps were filled completely with S247 dissolved in phosphate-buffered saline to concentrations corresponding to delivery of 1, 10 or 100 mg/kg/day per 20 g mouse. Mice receiving treatment prior to tumor cell inoculum were implanted with Alzet® Model 2002 (0.5 μl/hour, 14-day duration) one week before intracardiac injection. One week following injection, Model 2002 pumps were replaced with Alzet® Model 2004 pumps (0.25 μl/hour, 28 day duration). Post-injection treatment groups were implanted with Model 2004 pumps one week following intracardiac injection. Mice that showed signs of infection surrounding the pump or having lost the pumps were excluded from subsequent analyses since drug administration could not be properly controlled.

Metastasis assay

Immediately prior to injection, cells at 80 to 90% confluence were detached from 100-mm cell-culture plates (Corning, Acton, Massachusetts) using a solution of 2 mM EDTA and 0.125% trypsin in calcium- and magnesium-free Dulbecco's phosphate buffered saline (CMF-DPBS). Cells were counted using a hemacytometer, and resuspended in ice-cold Hank's balanced salt solution to deliver 2×10^5 viable cells in a volume of 0.2 ml. Cells were injected into the left ventricle of fully anesthetized (ketamine-HCl 129 mg/kg, xylazine 4 mg/kg), 3- to 4-week-old female athymic mice (Harlan Sprague-Dawley, Indianapolis, Indiana). Injections were performed using a 27-gg needle fitted onto a 1-ml tuberculin syringe. Immediately preceding and subsequent to inoculation, drawback of bright red arterial blood was used to verify that the injection was into the arterial compartment, as opposed to venous injections indicated by darker, burgundy-colored blood [47]. Food and water were provided *ad libitum* and animals were maintained under the guidelines of the National Institute of Health and appropriate Institutional Animal Care and Use Committees.

Mice were euthanized and necropsied five weeks post-injection. Bones were dissected free of musculature and soft tissues using a #21 scalpel blade and gauze or squares of paper towel to grip and remove remnants. Where possible, bones were left connected (e.g. femur–tibia–fibula, scapula–humerus–radius–ulna, ribcage–vertebrae) to facilitate orientation and to distinguish between sides. Dissected bones were then examined for metastases by fluorescence microscopy.

Fluorescence microscopy

To visualize metastases derived from the GFP-tagged cell lines, intact viscera and whole bones, dissected free of soft tissue, were placed in petri dishes containing CMF-DPBS to prevent drying and examined by fluorescence microscopy utilizing a Leica MZFLIII dissecting microscope with Plan 0.5× and PlanApo 1.6× objectives and GFP2 fluorescence

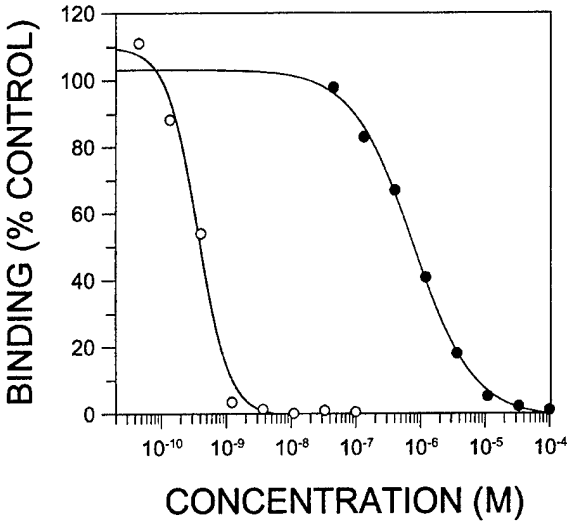


Figure 1. S247 is a potent $\alpha_{IIb}\beta_3$-sparing antagonist of $\alpha_v\beta_3$ integrin. Function of $\alpha_v\beta_3$ (○) and $\alpha_{IIb}\beta_3$ (●) was determined in the presence of increasing compound concentration as described in 'Materials and methods'. Representative data from a single experiment from each assay are shown on the same graph to simplify comparison. Each assay was performed five times producing an average $IC_{50} \pm$ standard deviation for $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ of 0.4 nM ± 0.24 and 380 ± 92, respectively. Similar IC50 values were obtained using a previously described $\alpha_v\beta_3$ solid phase receptor assay format (0.18 nM + 0.04) [44].

filters ($\lambda_{excitation} = 480 \pm 20$ nm, $\lambda_{emission} = 510$ nm barrier; Leica, Deerfield, Illinois). Photomicrographs were collected using a MagnaFire™ digital camera (Optronics, Goleta, California) and ImagePro Plus software (Media Cybernetics, Silver Spring, Maryland).

Mapping of bone metastases

During fluorescence microscopy, skeletal metastases were drawn on diagrams of murine bones (adapted from [53]). A custom computer program was written using Visual Basic 6 (Microsoft Corp., Redmond, Washington) in which the same diagrams were overlaid with a grid of squares ($\sim 0.30\ mm^2$). Metastases drawn for each mouse bone were transferred to the computerized grid. The program then calculated the percentage of mice in which tumor encompassed each square in the grid and depicted a composite image using color or grayscale. Composite images were then smoothed in Photoshop 6.0 (Adobe, San Jose, California) to reduce granularity.

Quantification of trabecular bone

Femurs and tibias were decalcified in 0.5M EDTA in CMF-DPBS for 24 h and paraffin-embedded [54]. One section from each of two levels that included an adequate representation of the distal femur trabecular region was stained with hematoxylin and eosin. Each distal femur (left and right) in each section was photographed under bright light using a Leica MZFLIII dissecting microscope with PlanApo 1.6× objective and MagnaFire™ digital camera. A matched image was then collected with the GFP2 fluorescence filter set. Under blue fluorescence, the eosinophilic calcified bone fluoresces brightly in the yellow-green spectrum, providing excellent contrast with the dark cartilage, marrow and tumor. Using ImagePro Plus software, tumor or normal marrow was first outlined on the bright-field H&E image. The outline was then transferred to the matching fluorescence image and the area of trabecular bone (i.e., not marrow) was automatically measured as bright objects within the outline. Finally, total area encompassed by the outline was quantified. The area of bone was then calculated. For trabecular bone quantification, only sections with no tumor in the distal femur were used.

Statistical analyses

Data were analyzed using SigmaStat 2.03 (SPSS Inc., San Rafael, California). Number of metastases for all groups were tested using Kruskal–Wallis one-way analysis of variance on ranks. Pair-wise multiple comparisons *versus* vehicle were performed using Dunn's method to determine which groups were significantly different from vehicle control. To determine statistical significance between the incidence of metastases, a Fisher's exact test was used to compare each treatment group with vehicle.

Results

S247 inhibits osteoclast actin ring formation

S247 inhibits $\alpha_v\beta_3$ integrin binding selectively over the related $\alpha_{IIb}\beta_3$ integrin (Figure 1), yielding IC_{50} values of 0.2 and 244 nM, respectively. These values are similar to those noted by Reinmuth et al. [45]. Early studies showed that osteoclasts exposed to S247 exhibited marked *in vitro* morphologic changes (Figures 2A, D). Reasoning that the change in morphology might reflect altered cytoskeletal organization and that osteoclast resorption of bone requires formation of an actin ring, the effect of S247 was assessed (Figure 2) in human and murine osteoclasts. There was a dose-dependent reduction of actin rings ($P < 0.05$). At the 30 μM dose, actin ring formation averaged 8.98% of control.

MDA-MB-435 cells were treated with S247 (0.01 to 1000 nM) to assess cytotoxicity. At doses up to 1000 μM, no decrease in viability was observed, but adhesion to vitronectin was inhibited in a dose-dependent manner (data not shown).

S247 inhibits bone metastasis

Mice were treated with S247 (1, 10, or 100 mg/kg/d) before and after intracardiac injection of MDA-MB-435^{GFP} breast carcinoma cells in order to begin assessing which step in the metastatic cascade was impacted by the drug [47]. Pretreatment would largely test whether S247 inhibited early (i.e., adherence and colonization) while post-injection treatment would assess later (i.e., proliferation and osteolysis) steps. A schematic diagram depicting the treatment schedule is depicted in Figure 3. Five weeks following tumor cell inoculation, mice were euthanized and tissues were collected. Bones were dissected free of soft tissue and the presence and

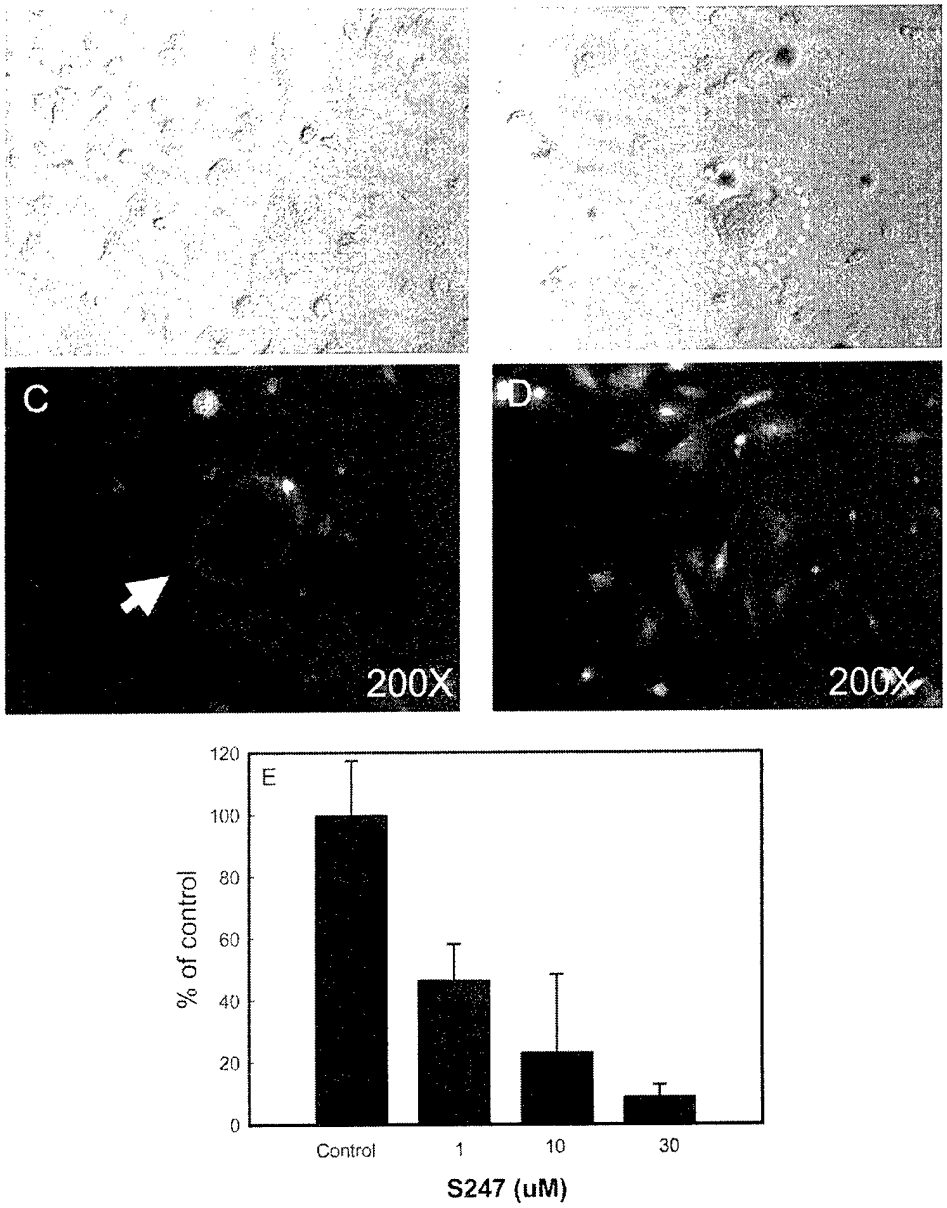
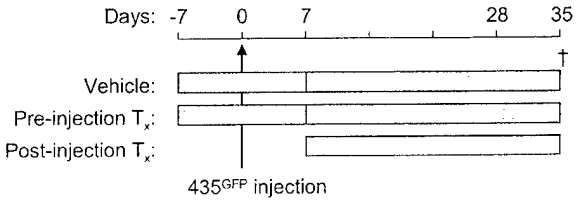


Figure 2. S247 alters osteoclast morphology and selectively inhibits formation of an actin ring in human osteoclast-like cell cultures. Osteoclasts grown on glass coverslips were treated for 4 h with S247 before assessment of actin staining with FITC-phalloidin or phalloidin-Texas red. (A) and (B) Phase contrast micrographs showing morphologic changes of osteoclasts in the absence (A) or presence (B) of S247. Actin ring formation was decreased in S247-treated cells (D) compared to saline-only treated osteoclasts (C). The number of cells forming actin rings in S247-treated cultures were counted and compared to control (saline treated) cells (E).

size of fluorescent bone and visceral metastases were recorded using a fluorescence stereo microscope. The status of the implanted pump was also noted at necropsy. Although data were collected on all animals, five mice exhibiting signs of inflammation around the pump or which showed signs that the pump implant was compromised were excluded from data analysis (note: mice in the pre-treatment groups were not excluded if the pump was lost after tumor cell injection).

Fluorescence microscopy revealed a significant ($P = 0.002$) dose-dependent inhibition for both incidence and number of metastases among pre-injection treatment groups (Figures 4 and 5). For ease of comparison, femur and tibia metastases, which generally reflect skeletal-wide incidence, will be used below. Mice pre-treated with S247 had incidences of bone metastases at 50%, 30% and 25% compared to vehicle-only-treated mice for the 1, 10 and 100 mg/kg/day groups, respectively. By contrast, mice treated with S247 after tumor cell inoculation were modestly lower than vehicle-only-treated mice (63%, 82% and 63%, respectively). Compared to vehicle-treated animals, only



Groups	S-247 (mg/kg/day)
1. Control:	-
2. Vehicle:	-
3. Pre-injection T_X:	1
4. "	10
5. "	100
6. Post-injection T_X:	1
7. "	10
8. "	100

Figure 3. Schematic representation for *in vivo* analysis of S247 effects on breast cancer metastasis to bone. S247 treatment (Tx) using mini-osmotic pumps began one week prior to, or one week after, inoculation of 435^{GFP} cells (2×10^5). Mice were euthanized and necropsied five weeks following tumor cell injection and examined for fluorescent skeletal metastases (†).

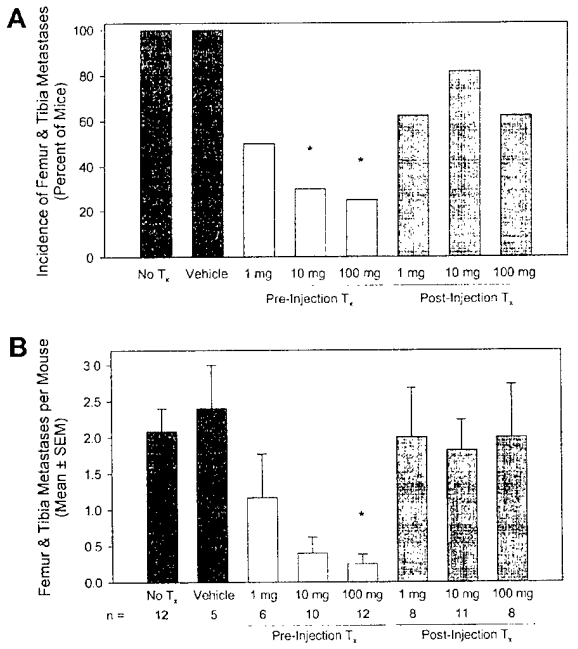


Figure 4. Incidence and number of 435^{GFP} femur and tibia metastases are significantly ($P = 0.002$) reduced in S247 pre-treated mice. Female athymic mice were treated with S427 (1, 10 or 100 mg/kg/day) starting either one week prior to (pre-injection T_X) or one week subsequent to (post-injection T_X) intracardiac injection of 2×10^5 435^{GFP} cells. Mice were euthanized and necropsied five weeks after tumor cell injection. Fluorescent metastases in intact bones were recorded. (A) Percent of mice exhibiting at least one metastasis in a femur or tibia. (B) The number (mean ± SEM) of metastases to femur and tibia per mouse. *Significantly different from vehicle.

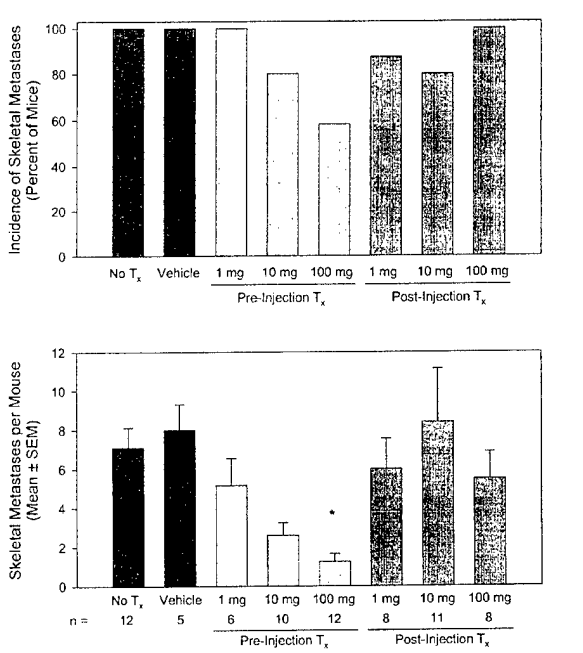


Figure 5. Overall number of 435^{GFP} skeletal metastases are significantly ($P = 0.002$) reduced in a dose-dependent manner following pre-treatment of mice with S247. Mice were treated as depicted in Figure 1. (A) Percent of mice having at least one skeletal metastasis (includes: skull, mandible, ribs, vertebrae, scapula, humerus, radius, ulna, pelvis, femur and tibia.). (B) The number (mean ± SEM) of skeletal metastases per mouse. *Significantly different from vehicle.

pre-treatment with 100 mg/kg/d S247 significantly inhibited incidence and number of hind limb bone metastases.

Bone metastases localized predominantly to trabeculae in proximal and distal femur and proximal tibia (Figure 6A). In addition to inhibiting the number of metastases, pre-treatment also appeared to limit metastatic burden (i.e., size of metastases).

Quantifying osteolysis

To determine if treatment with S247 inhibited osteolysis, distal femoral trabecular bone area was quantified in H&E stained histological sections for mice receiving 100 mg/kg/d S247 and compared to mice receiving either no treatment or treatment with vehicle only (Figures 6B–E). The trabecular bone area in tissue encompassed by 435^{GFP} metastases was compared to the area of trabecular bone where no tumor was evident. As expected, there was a trend toward decreased bone area (i.e., osteolysis) when tumor cells were present (Figure 6F); however, the difference was not statistically significant. Tumor cells were not encountered in sections examined from pre-injection group. Thus, despite evidence that 435^{GFP}-induced osteolysis, this method was not sufficiently robust to quantify trabecular bone resorption at five weeks. More elaborate histomorphometric methods would be required. In general, then, osteolysis (decrease in trabecular bone area) in the 100-mg post-injection group was

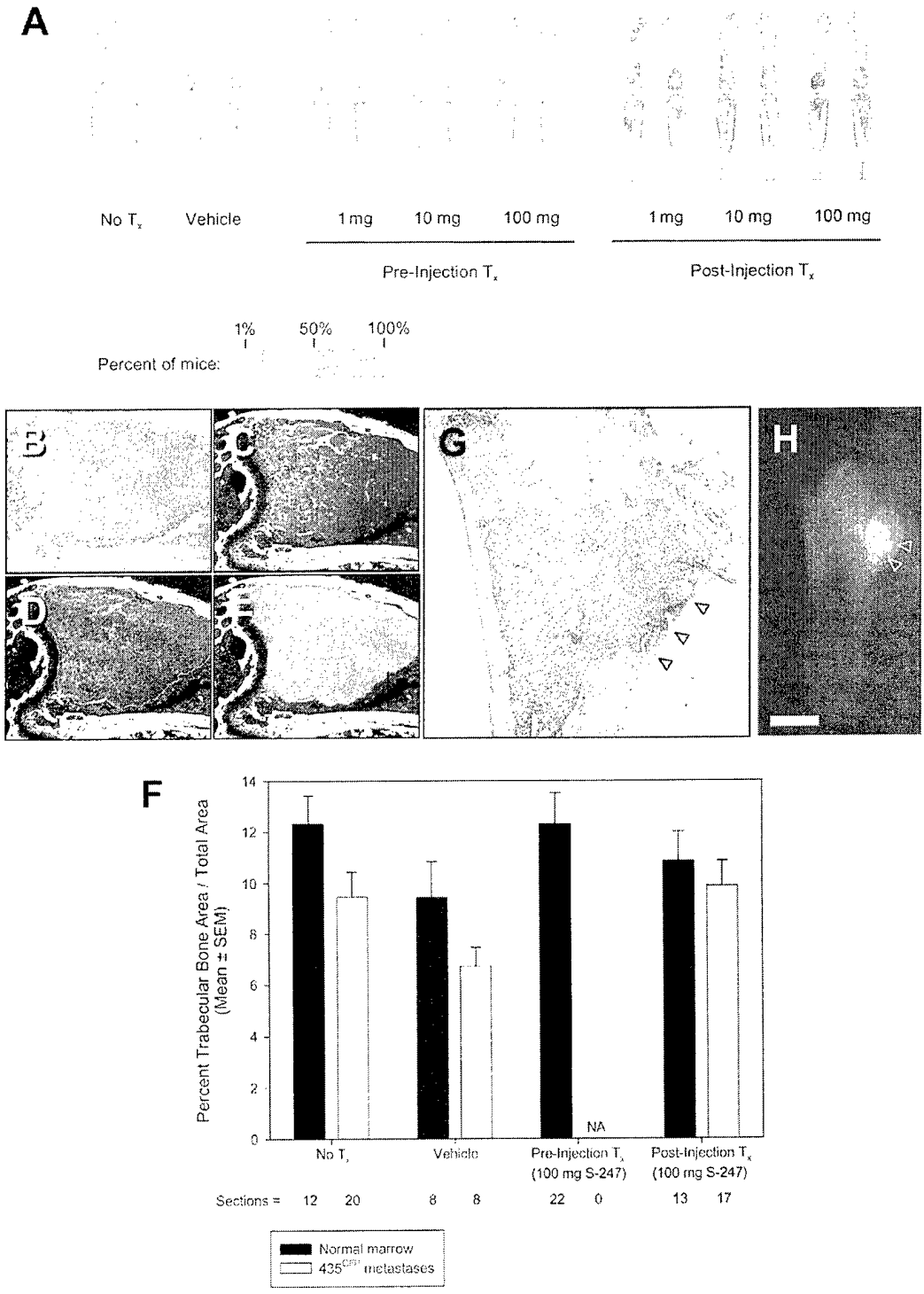


Figure 6. Metastatic tumor burden in S247 pre-treated mice is decreased. As expected, bone metastases localized predominantly to trabeculae in femurs and proximal tibia. Fluorescent (A) metastases were diagramed by hand on figures represented above. Computer software was used to calculate area encompassed by tumor (approximate resolution = 0.33 mm^2). Trabecular bone area of distal femur was estimated using H&E-stained sections photographed under bright light (B) and fluorescence (C). under which eosinophilic calcified bone brightly fluoresces. Metastases or normal trabecular marrow were outlined on bright field images and the percent area of trabecular bone (D) was calculated relative to the total area (E). (F) Generally, trabecular bone area decreased within metastases, consistent with osteolysis. But the differences were not statistically significant. The decrease (osteolysis) evident in S-247-treated mice (post-injection T_X) is less than control groups. No metastases were encountered in random sections from S247-pre-treated bones. (G) H&E-stained histological section of proximal femur from a mouse treated post-injection with 100 mg/kg/d S-247. Note considerable osteolysis of cortical bone in the region leading to the ball of the femur (arrowheads). (H) Fluorescence image of the same femur freshly dissected depicts the fluorescent 435^{GFP} metastasis and reveals bright foci corresponding to significant degradation of overlying cortical bone (arrowheads). Bar = 1 mm.

present, but somewhat less than control groups (Figures 6G, 6H).

S247 inhibition of non-osseous metastasis

Soft tissues were also examined for metastases. Except for brain, in which a sagittal bisection was performed, fluorescent metastases were quantified in intact organs as previously described [47, 54]. Results were consistent with previous studies showing non-skeletal 435^{GFP} metastases were found most frequently in adrenal glands, brain and ovary. A slight dose-dependent decrease in the incidence of adrenal and brain metastases was seen in pre-injection treatment groups Figure 7. No metastases to ovary were detected with any S247 treatment regimen. However, despite these trends, none of the differences in soft-tissue metastasis were statistically significant from controls. The relatively low incidence of 435^{GFP} metastasis to these sites does not provide the statistical power needed to determine an effect within the size of these experiments.

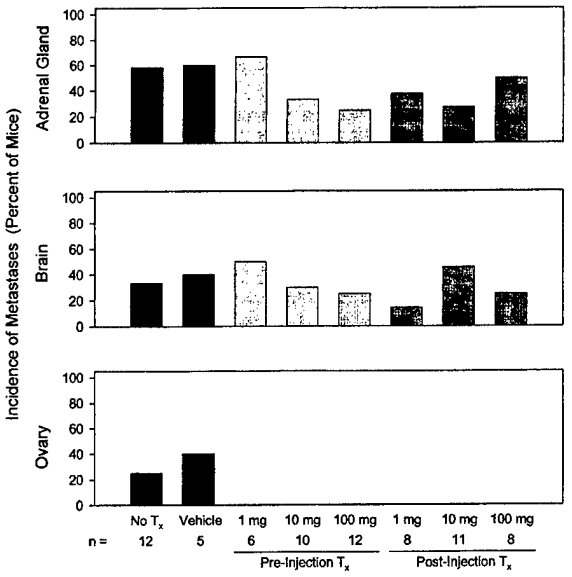


Figure 7. Inhibition of extra-osseous metastases (adrenal gland, brain and ovary) by S247 is inconclusive. Despite the absence of ovary metastases in S247-treated groups and a dose-dependent inhibition in pre-injection treatment groups, the suppression is not statistically significant.

Discussion

When cancers are confined to breast, long-term survival rates are high [55]. But when cancer cells metastasize, cure rates drop precipitously. Skeletal metastases are common, particularly for breast, prostate and myeloma tumors. For these tumor types, incidence of bone metastases is greater than colonization elsewhere. Approximately 75% of women with breast cancer develop bone metastases while only a third develop metastases to lung and/or liver [56]. While metastases to bone are not the most lethal, they are significant because: (i) they can be a harbinger for metastases in vital organs; (ii) patient quality of life is poor; and (iii) the sequelae of breast cancer metastases in bone represent the reason for roughly two-thirds of treatment costs [57]. Therefore, a better understanding of the underlying mechanisms responsible for breast cancer tropism for bone and the subsequent osteolysis would be helpful at many levels.

Based upon its previously demonstrated roles in breast cancer metastasis and osteolysis, we hypothesized that the $\alpha_v\beta_3$ integrin may control, in part, metastasis to bone. To test this hypothesis, we used a specific small molecule inhibitor of $\alpha_v\beta_3$ binding, S247. When the drug was present prior to tumor cell inoculation into the left ventricle of the heart, the number of osseous metastases was significantly decreased in a dose-dependent manner. Likewise, the overall tumor burden, as indicated by area of fluorescence or size of lesions in representative in histologic sections, was decreased. Mice receiving S247 after tumor cell inoculation showed a slight trend toward decreased metastasis to bone; however, the inhibition was not statistically significant.

The results demonstrate that $\alpha_v\beta_3$ integrin is indeed important in the development of osteolytic bone metastases in some breast carcinomas. Since the inhibition was observed if S247 was present at the time tumor cells entered the vasculature but not if present after tumor cells were inoculated, we infer that $\alpha_v\beta_3$ is more critical for early arrest and/or survival of cells rather than later steps in the metastatic cascade (i.e., colonization, proliferation). However, the inhibition may relate to interactions between $\alpha_v\beta_3$ and matrix metalloproteinases that can regulate metastasis [58], although this aspect was not evaluated in this study.

Such results suggest that S247 might be useful in preventing development of bone metastasis. However, the practicality of metastasis prevention in a clinical setting is lower than elimination of already-established lesions. Nonetheless, there are situations in which prevention of progression would be warranted.

Previous studies have shown that S247-treated mice were inhibited for osteolysis; therefore, we anticipated that the drug would similarly inhibit breast carcinoma-induced bone resorption. It is well-established that breast carcinoma cells in metaphyseal areas secrete PTHrP and other factors that induce bone resorption [59–61]. Here we showed that treatment with S247 was associated with disruption of the sealing zone around osteoclasts *in vitro*. Thus, it was speculated that S247 might inhibit bone metastasis both by blocking tumor cells and by inhibiting osteoclasts. Unfortunately, at the time examined, S247 treatment resulted in only a modest decrease in osteolysis when assessed histologically or by micro-computerized tomography (data not shown). Additional experiments will be required to determine direct effects of $\alpha_v\beta_3$ inhibitors on breast cancer osteolysis. In summary, our results show that inhibition of $\alpha_v\beta_3$ integrin interactions represent a promising target for controlling breast cancer metastasis to bone.

Acknowledgements

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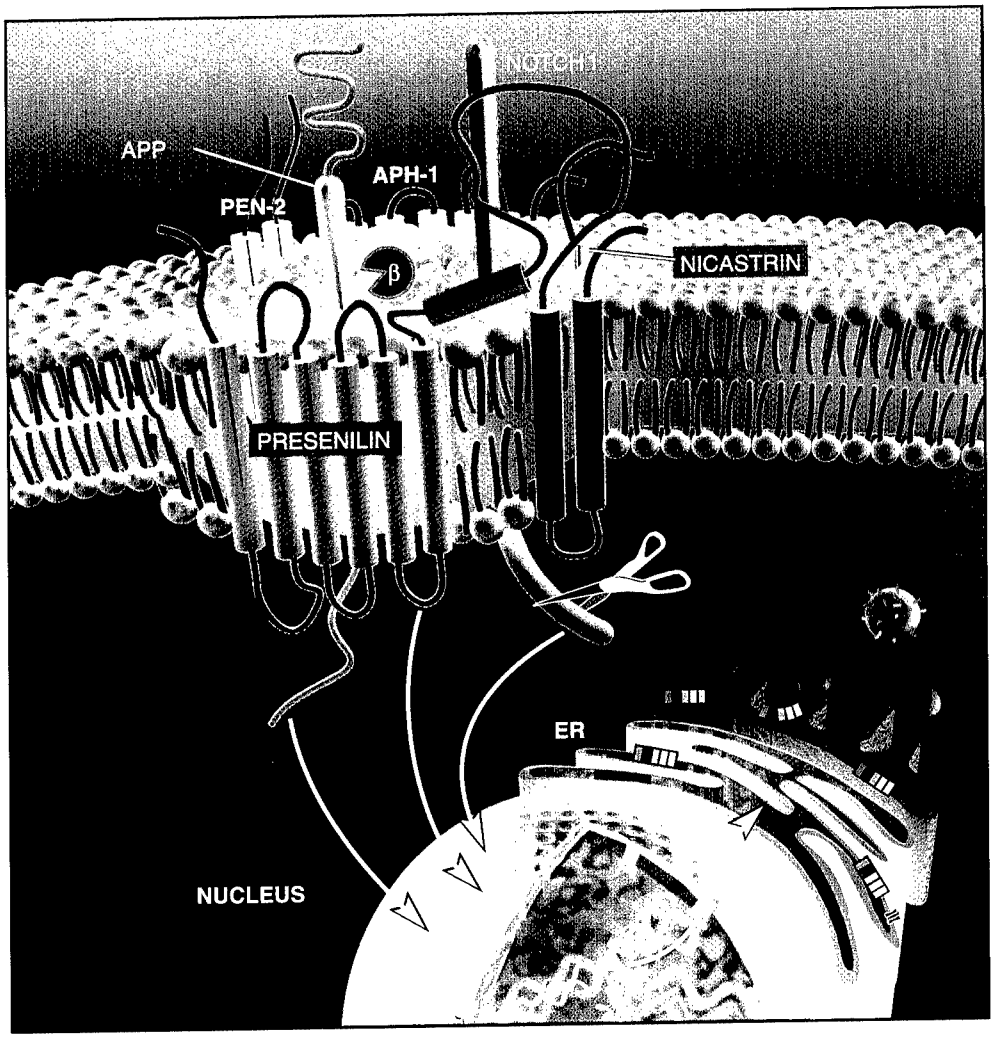

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Metastasis Regulatory Genes

Danny R. Welch

When cancer cells spread and colonize other tissues, patient survival is diminished. Yet, while millions of cancer cells migrate from primary tumors daily, only a tiny fraction of those cells successfully colonize and proliferate at secondary sites. Accumulating evidence has identified a growing number of genes that specifically control the metastatic phenotype without affecting tumorigenicity. Mutations to these genes allow migrating neoplastic cells to colonize distant loci, where the neoplastic cell then must interact with and modulate the local cellular microenvironment in order to proliferate. Discovery of metastasis suppressor genes and the context in which those genes function may allow opportunities to interfere in the metastatic cascade.

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Metastasis culminates a process of neoplastic evolution in which individual transformed cells generate mutants, some of which are able to disseminate and colonize secondary sites. Although DNA damage occurs frequently in normal cells, these cells either repair the defects or exit the proliferative pool (i.e., undergo apoptosis). Typically, less than one mutation arises for every billion epithelial cell divisions in normal human cells.

In contrast, transformed cells often exhibit mutation rates 1000- to 10,000-fold higher than their normal counterparts. Some cancer cells have shown mutation rates more than 10 million-fold higher than normal cells.

This genomic instability is a hallmark of cancer cells and is presumably the driving force for tumor progression. New variants are subjected to selective pressures so that, according to Darwinian principles, the "fittest" prevail. While the occurrence of one or several genetic mutations is believed necessary for the transformation of normal cells into neoplastic ones, the continued accumulation of mutations is needed to allow these cells to disseminate and grow in distant foci.

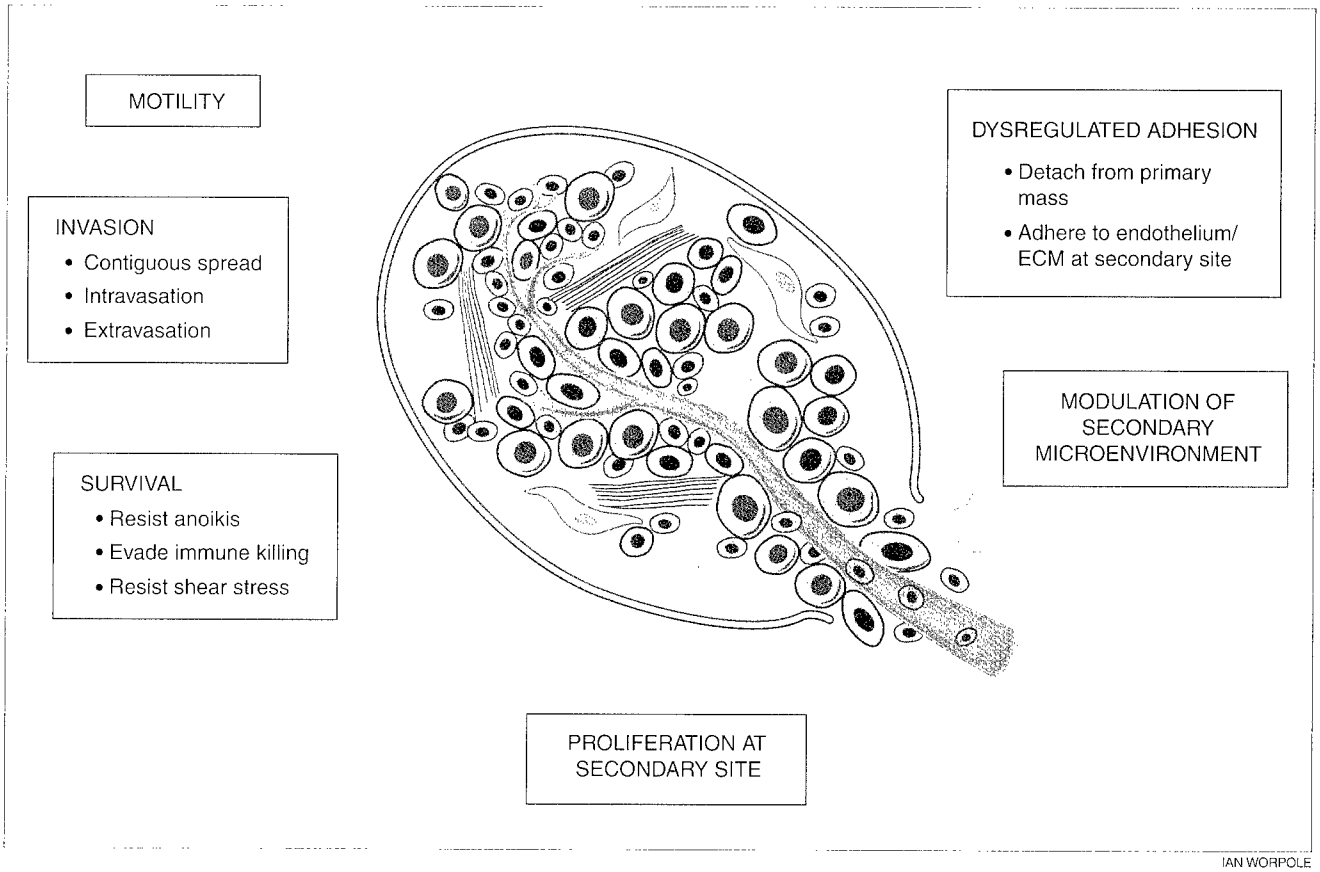
A variety of oncogenes have been identified that, when mutationally dysregulated or abnormally overexpressed, contribute to tumorigenicity. Likewise, over the last 15 years, tumor-suppressor genes have been identified whose products act to block tumor development and proliferation. In normal cells, these genes function in cell cycle checkpoint control, mitogenic signaling, protein turnover, DNA repair, and other stress responses, and their loss enables tumor cell growth.

More recently, a small number of genes has been associated specifically with metastatic growth. Identification and characterization of metastasis suppressor genes may yield novel targets for chemopreventive or antimetastatic therapies. This article reviews the discovery of these genes and the context in which they function.

Metastatic Potential is a Distinct Phenotype

Neoplastic cells exhibit several hallmarks besides genomic instability—immortality, abnormal growth (i.e., self-sufficient growth that is often insensitive to growth inhibitory signals), dysregulated cell cycle, evasion of programmed cell death, and sustained angiogenesis. Yet, not all neoplastic cells are metastatic. Metastatic cells are distinguishable from ordinary neoplastic cells because they have acquired the ability to migrate, invade, and colonize secondary sites.

Millions of tumor cells migrate away from the primary tumor mass and are shed into the vasculature daily (1 to 4×10^6 cells/g of tumor/day). Fortunately, a vanishingly small percentage of cells that enter



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a circulatory compartment successfully colonize secondary sites. This inefficiency highlights an important point—migration and invasion are necessary, but not sufficient, for metastasis.

In other words, invasion and metastasis are distinct phenotypes. This contention is illustrated by highly invasive tumor types that rarely develop distant metastases, such as basal cell carcinoma of the skin or glioblastoma.

Circulation and Invasion Are Parts of a Metastatic Cascade

After tumor cells enter the circulation, the events that occur are largely uncertain, because technologies to study these steps of the metastatic cascade are only recently becoming available. As a result, some discrepancies exist. Several scenarios likely will be operant, depending on the tumor type and host context.

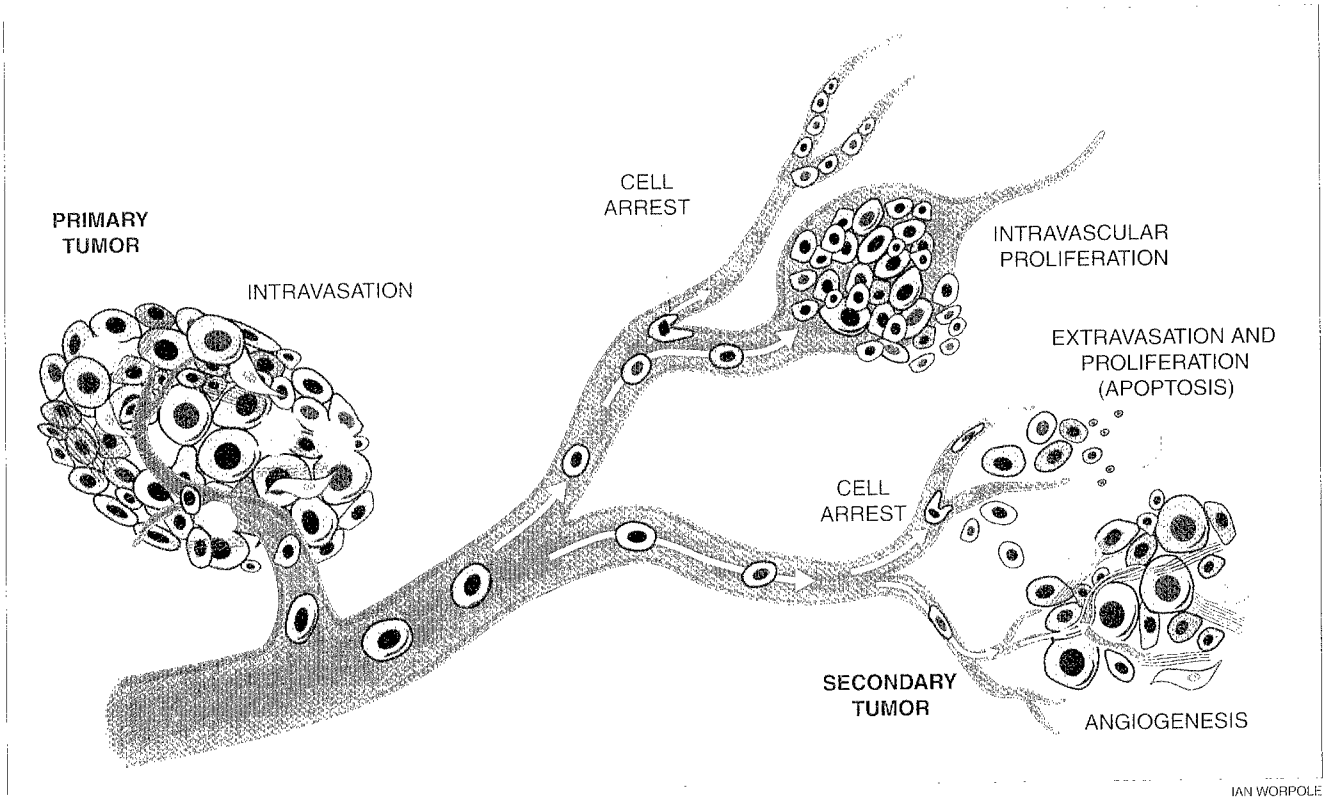
From early studies using radiolabeled tumor cells, it was believed that extremely small percentages of tumor cells entering the circulation could survive hemodynamic shear forces. However, recent studies with intravital microscopy have found that substantial percentages can survive in the circulation.

Once cells arrest in the circulation, there are conflicting models about the events that can produce metastases. Ann Chambers, Alan Groom, and colleagues at the University of Western Ontario have shown that a majority of tumor cells in the circulation can extravasate into surrounding tissue. In contrast, Ruth Muschel and colleagues, in experiments with their pulmonary metastatic cell lines, did not find extravasation of tumor cells but rather proliferation within the vessels.

Both scenarios are likely to occur in human cancers, and the relative frequency of each affects how therapeutics can be developed to prevent or treat metastasis.

Dissemination of tumor cells can occur also via nonhematogenous routes, such as the lymphatics or

Metastatic cells are a subset of neoplastic cells. In addition to the characteristics that allow cells to become malignant (i.e., defined as invasion through a basement membrane), tumor cells also must demonstrate motility, the ability to detach from a primary mass yet reattach at the endothelium or extracellular matrix (ECM) at secondary sites. Tumor cells also must survive in the circulation, evade immune cell killing, and resist shear forces. At the secondary site, tumor cells then modulate the secondary tissue environment, where the tumor cells begin to proliferate to form metastatic lesions.



Metastasis begins with primary tumor growth and the accumulation of genetic changes. Tumor cells respond to microenvironmental signals that enable them (temporarily or permanently) to complete subsequent steps of the metastatic cascade. For tumors to grow larger than 1 mm in diameter, they must respond to hypoxia with angiogenesis. Tumor cells invade local tissue, penetrate a basement membrane and eventually enter a vessel (intravasation).

Circulating tumor cells must survive in the circulation, where at times they may be surrounded by platelets, fibrin, leukocytes, or other tumor cells. Large homo- or heterotypic emboli are effectively trapped as vessel diameters decrease. Alternatively, tumor cells can adhere selectively to endothelium or ECM components. Whether tumor cells proliferate inside vessels or extravasate is dependent on the tumor cell and the tissue into which the cells migrate.

At the secondary site, tumor cells proliferate but may become dormant or die. Evidence suggests that most metastases are clonal in origin. Metastases can repeat this process to form tertiary or quaternary lesions as well.

Intravital microscopy *is a collection of methodologies, usually involving a fluorescent molecular probe (e.g., GFP) and a microscope equipped with a detection system (e.g., two-photon laser microscopy), which is used to provide direct noninvasive imaging of molecular and cellular processes in intact living tissue, either in situ or in vivo.*

body cavities. The route determines, in part, the site of metastases.

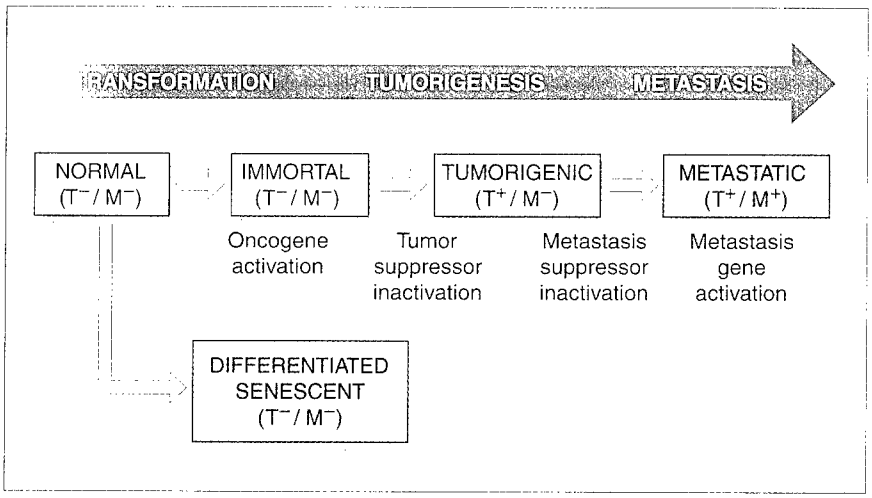
Substantial proportions of tumor cells arrest in the first capillary bed encountered, but they do not always proliferate at the site of initial arrest. Likewise, some cells pass through tissues readily and arrest selectively using tissue-specific endothelial cell surface molecules.

Such differences in cellular behavior partly explain organotropism of metastases. Mere dissemination and arrest do not constitute metastasis. Metastasis requires that tumor cells proliferate in the secondary tissue, a process termed *colonization*.

Therefore, metastatic cells must complete every step of the metastatic cascade. If a cell is deficient for any step of the process, it is nonmetastatic.

Because each step of the metastatic cascade is rate limiting and because all metastatic capacities must be present within each cell, confusion regarding interpretation of microarray data comparing bulk primary tumors and metastases (not microdissected tumor cells, but tumor cells mixed with stromal cells) has developed.

In brief, several microarray studies have shown that mRNA expression patterns of primary and metastatic cells are similar, leading some to conclude that metastatic competence is acquired early in tumor progression. However, the inefficiency of the metastatic pro-



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Tumor progression involves at least four broadly defined genetic changes. Conversion from a normal to a transformed, but not yet tumorigenic, phenotype involves the loss of genomic stability and loss of senescence gene functions. Tumorigenicity (T^+) occurs by activation of oncogenes and inactivation of tumor suppressor genes, whereas conversion from a tumorigenic/nonmetastatic (T^+/M^-) to a tumorigenic/metastatic phenotype (T^+/M^+) broadly involves the activation of metastasis-promoting genes and the inactivation of metastasis-suppressing genes.

Tumor suppressors are distinct from metastasis suppressors. Metastasis suppressors block metastasis but not tumorigenesis, whereas tumor suppressors block both metastasis and tumorigenicity since tumorigenicity is prerequisite to metastasis.

cess and the requirement for cells to complete multiple steps before successful metastasis make this conclusion counter to decades of biological and genetic data.

As an analogy, consider a salvage yard, which contains thousands of nonfunctioning automobiles. In the salvage yard, perfectly functional parts (e.g., engine, brakes, drive train, etc.) can be found. The functional parts, however, do not form an operational vehicle because they are not assembled as a single unit. Cars can function only when all of the parts are properly assembled. Automobiles may differ in size, style, engine, specifications, or fuel efficiency, yet each inherently incorporates all of the functional requirements that characterize it as an automobile.

Like the cars in the salvage yard, subpopulations of tumor cells have some of the characteristics necessary to metastasize, but most do not have all of the characteristics. Thus, they can invade but cannot survive in the circulation, or proliferate at secondary sites, or are susceptible to immune killing, and so on.

Metastatic Ability Requires Coordinated Expression of Multiple Genes

The requirement for coordinated expression of multiple genes for metastasis is elegantly highlighted by recent data from the laboratories of Joan Massagué and Theresa Guise. Briefly, they isolated a variant subpopulation of human MDA-MB-231 breast carcinoma cells that colonize bone efficiently. Transcriptomes were compared between parental MDA-MB-231 cells and a bone metastasis-selected variant (231-bone).

The authors identified 43 overexpressed and 59 underexpressed genes. This pattern was defined as a "bone metastasis signature."

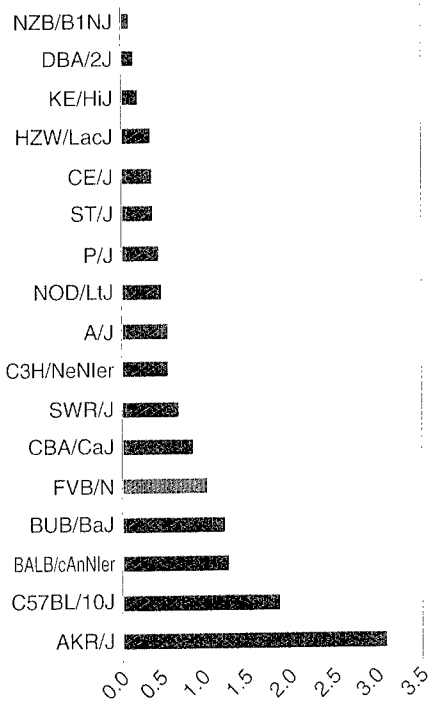
Among the overexpressed genes were the matrix metalloproteinase MMP-1, osteopontin (OPN), interleukin-11 (IL-11), the chemokine receptor CXCR4, and connective tissue-derived growth factor (CTGF). Subpopulations within the parental population expressed one or more of the bone signature genes, but only a few expressed all of them.

Co-transfection of gene combinations into the parental 231 cell line resulted in populations as efficient at bone colonization as the 231-bone variants, whereas transfection of individual cDNAs only modestly increased bone metastatic efficiency.

For example, transfection of OPN or IL-11 cDNAs alone resulted in modest increases in metastasis. However, co-transfection of OPN or IL-11 with CXCR4, CTGF, and MMP-1 produced bone colonization.

These data, as well as those from similar experiments, illus-

See the paper by John Chirgwin and Theresa Guise, entitled "Cancer Metastases to Bone," in the June 2003 issue of Science & Medicine, for a discussion of tumor-bone interactions that enhance metastatic growth.



Metastatic ability of the PyVT tumor varied when bred in mice of different genetic backgrounds. Homozygous FVB/NJ mice carrying the PyVT oncogene (*red bar*) were bred with different maternal strains (*x-axis*). The variation in metastasis development suggests that subtle genetic differences between strains affect the metastasis process.

trate several points:

- First, there are specific genes that control metastasis. Coordinated expression of multiple genes is required for metastases development.
- There is a hierarchy of gene expression in cancer cells. Not only does gene expression determine metastatic capacity, but some genes determine where tumor cells can colonize.
- Many of the genes differentially expressed are known to be involved in cellular responses to exogenous signals (e.g., IL-11, CXCR4, CTGF), highlighting the importance of tumor-stromal interactions.
- Finally, gene regulation in metastatic cells is, in part, dependent on cellular context—i.e., where the cell is and what signals are impinging on it.

Tissue Microenvironment Also Influences Metastatic Cells

The importance of cellular context has emerged as the next fertile field for research. At the National Cancer Institute, Kent Hunter and colleagues have demonstrated this concept elegantly.

In brief, transgenic mice expressing the polyoma middle T oncogene under control of the mouse mammary tumor virus promoter develop metastatic mammary tumors. When the transgenic mice were bred to nonsyngeneic mice, the metastatic potential was enhanced or inhibited, depending on the strain of mouse.

Because all tumors were initiated by the same oncogenic event, differences in metastasis development are most likely explained by differences in genetic background rather than the nature of the oncogenic event.

Just as carcinogenesis can vary depending on the background strain, similar data are emerging for metastasis. Ultimately, one can conclude that both tumorigenesis and metastasis involve both inherent genetic components as well as cellular responses to extrinsic stimuli.

How does the microenvironment at a metastatic site affect metastatic cell behavior? Interactions are reciprocal and reflect endocrine, paracrine, juxtacrine, matrix composition, and immunologic status.

Even specific cell types can stimulate or inhibit steps of the metastatic cascade. For example, infiltrating macrophages can eliminate tumor cells, promote angiogenesis, and/or promote invasion. Indeed, recent data suggest that proteinases in the tumor milieu are largely stroma-derived.

Two examples illustrate the proinvasive and prometastatic activities of immune cells. Eli Gorelik and colleagues in 1982 were the first to show that activated macrophages enhanced local invasiveness of B16 murine melanoma cells. Subsequently, Paul Aeed, Dan Schissel, Rick Howrey, and I found that tumor cells elicited a specialized

GENETIC IDENTIFICATION METHODS

- **Comparative genomic hybridization (CGH)** — Use of fluorescent genetic probes to compare chromosomal content of cells by competitive hybridization to chromosomal spreads
- **Loss of heterozygosity (LOH)** — Loss of an allele in a cell as assessed by the lack of the heterozygosity that would be expected based on inheritance of alleles from each parent
- **Karyotype analysis** — Analysis of chromosome structure and number using probes (fluorescent) or stains that identify banding patterns
- **Microcell-mediated transfer (MMCT)** — Introduction of a chromosome (whole or part) into a cell via cell fusion
- **Differential hybridization** — PCR-based method to compare mRNAs produced by two or more cell populations
- **Subtractive hybridization** — Method to isolate mRNAs expressed by one cell population that are differentially (lower or higher) expressed by a second population

subset of neutrophils that would enhance invasiveness and metastasis of rat mammary adenocarcinoma cells by up to 25-fold. Carl McGary later showed that tumor cells secreted GM-CSF and IL-3-like molecules to recruit and activate the neutrophils.

Thus, metastatic cells modify the host environment so that tumor cells are nurtured, although the specific mechanisms by which tumor cells manipulate their microenvironment (and vice versa) are not well understood.

Metastasis Suppressor Genes Can Be Identified by Several Methods

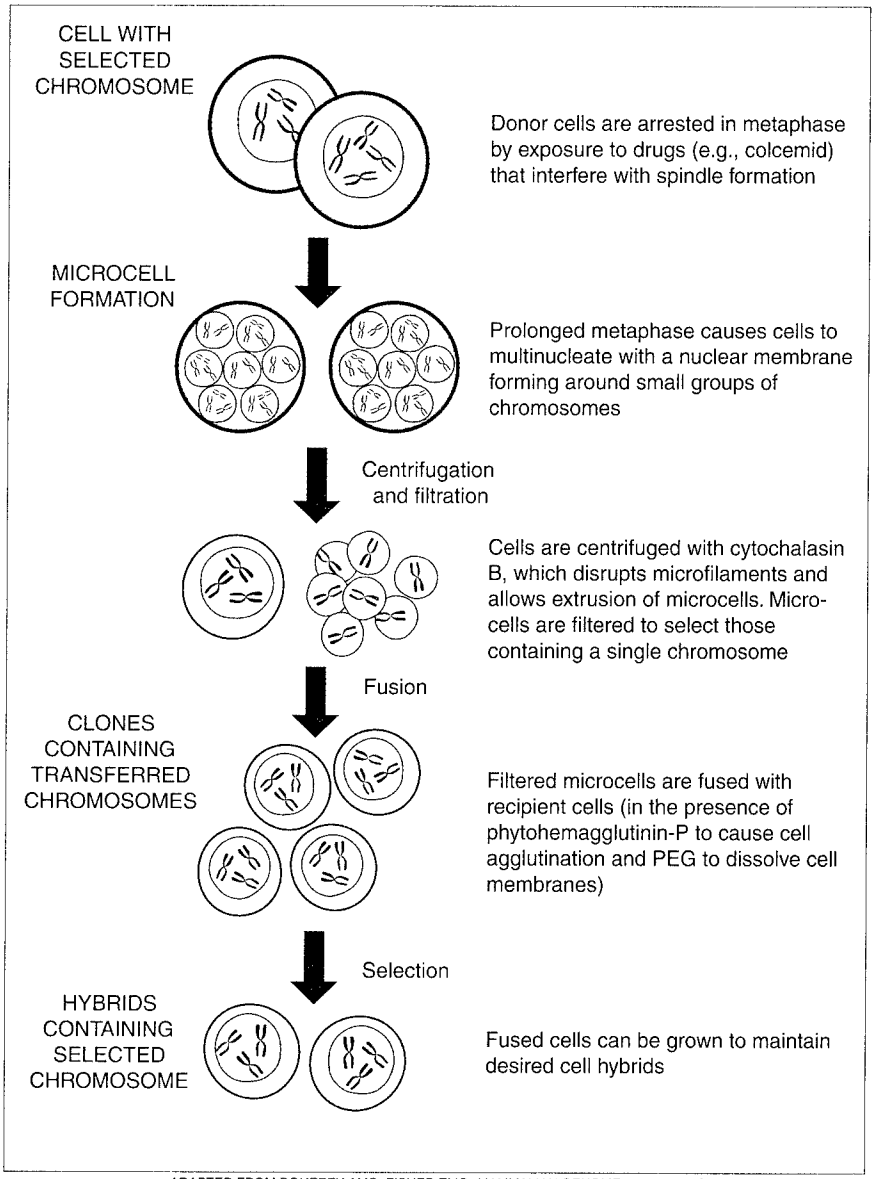
So far, the examples noted here have focused on the coordinated expression of genes that *enable* metastasis. However, other genes block metastasis. Whereas it takes a finely orchestrated set of genes to produce metastasis, blockage of any single step of the metastatic cascade can halt the process. Identification of these inhibitor genes may lead directly to therapeutic interventions.

Various studies using comparative genomic hybridization, loss of heterozygosity, and karyotype analyses have identified distinctively altered regions and/or genomic imbalances involving various human chromosomes. Some of these changes correlated temporally with acquisition of metastatic capacity. By inference, then, these chromosomal regions were suspected to harbor metastasis-suppressor-associated genes.

In the case of genetic loss, replacement of the chromosomes by microcell-mediated chromosomal transfer (MMCT) was predicted to suppress metastasis. MMCT of chromosomes 2, 7, 8, 10, 11, 12, 13, 16, 17, and 20 suppressed metastasis of prostate carcinoma cells without blocking tumorigenicity.

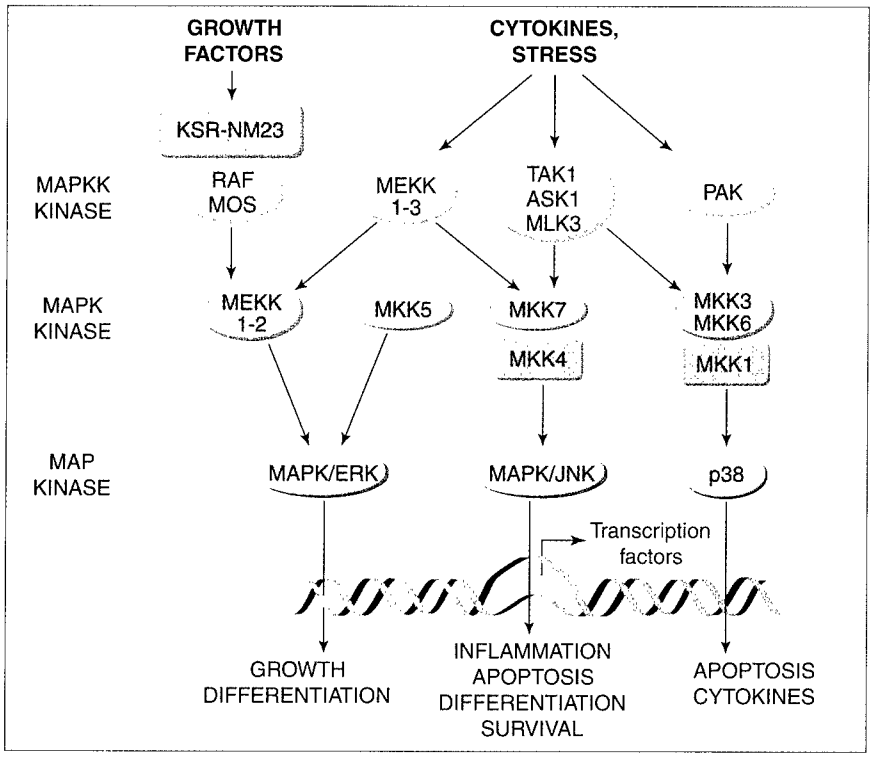
In subsequent experiments, Carrie Rinker-Schaeffer and colleagues, at the University of Chicago, used positional cloning to identify *MKK4* on chromosome 17 as a putative metastasis suppressor gene, and Jin Tang Dong and colleagues, at Johns Hopkins University, identified *KAI1* on chromosome 11.

In a study using human metastatic melanoma cells, MMCT of human chromosome 6 completely suppressed metastasis. Differential display and microarray studies were then used to identify *KISS1*, *TXNIP*, and *CRSP3* as putative metastasis-suppressor genes. Likewise, in human breast carcinoma cells, MMCT of chromosome 11 suppressed lung and lymph node metastases, and differential display was then used to identify *BRMS1*.



ADAPTED FROM DOHERTY AMO, FISHER EMC: *MAMMALIAN GENOME* 14:583-592, SEP 2003; IAN WORPOLE.

Developed in the mid-1970s, MMCT is a technique of cell fusion that uses a series of manipulations to produce microcells from a donor cell line, fusion into a recipient cells line, and then selection of hybrids containing the chromosome of interest.



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Metastasis suppressors affect the mitogen-activated protein kinase (MAPK) signaling pathway. In normal circumstances, various stimuli initiate the arms of the pathway by the serine/threonine phosphorylation of an MAPK kinase kinase (*gray*), which in turn phosphorylates tyrosine/threonine residues of an MAPK kinase (*blue*) which then phosphorylates and activates an MAPK (*green*). The activated MAPKs then activate transcription factors, leading to cellular responses that are important to cancer and metastasis. Metastasis suppressors (*purple*), such as NM23 and MKK4, bind intermediates in the pathways, inhibiting subsequent signaling.

membrane protein family. which are widely expressed and have diverse functions.

Expression of this gene in epithelial cells is consistently downregulated in prostate, breast, uroepithelial, gynecologic, and lung cancers. Re-expression in breast, prostate, and melanoma xenografts results in decreased metastasis.

KAI1 expression during tumor progression appears to be reduced, similar to that seen with other metastasis suppressors. Sequences of the metastasis suppressor genes usually are not mutated, but mutations in the promoter region or methylation of CpG islands has been observed, suggesting that upstream regulators may be critical to the activity of these genes.

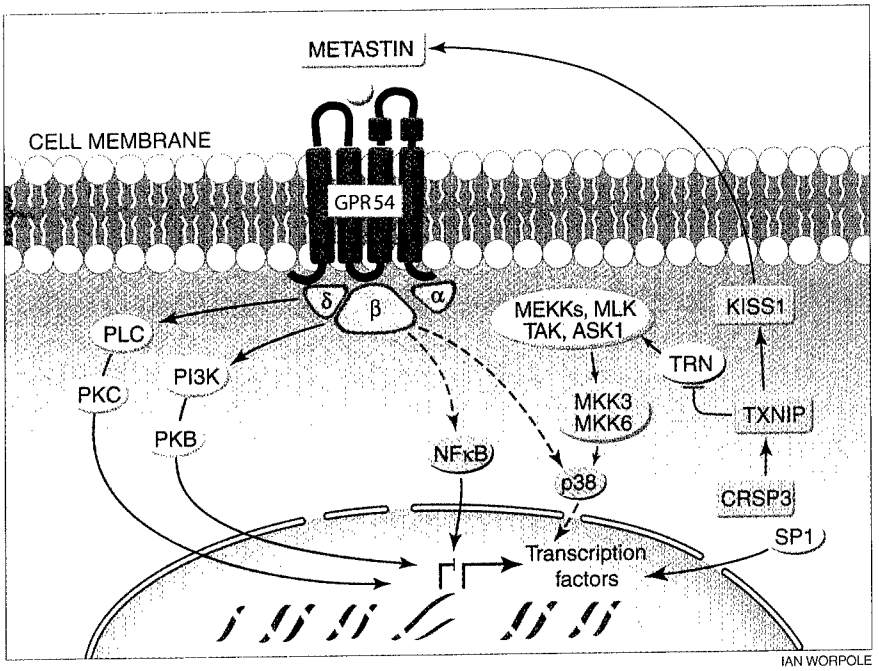
The mechanism of action of *KAI1* remains unknown. KAI1 is an adhesion molecule on leukocytes but does not dramatically influence tumor cell adhesion. KAI1 also directly associates with epithelial growth factor receptor (EGFR) and suppresses induced lamellipodia and migration signaling. It also associates with specific guanine exchange factors and adaptor proteins, suggesting a role in signaling.

KISS1, TXNIP, and CRSP3

KISS1 was identified by subtractive hybridization by comparing a metastatic C8161 human melanoma cell line with metastasis-suppressed MMCT chromosome 6 hybrids. *KISS1* was mapped to chromosome 1q32, but a deletion variant [neo6del (q16.3-q23)] of neomycin-tagged human chromosome 6 did not suppress metastasis and did not express *KISS1*. Thus, regulators of *KISS1* were hypothesized to be encoded at 6q16.3-q23.

The mechanism of action of *KISS1* is still unknown. Research has been stymied by a short protein half-life (« 1 min). Nonetheless, three groups have identified an amidated fragment(s) of *KISS1* as the ligand for an orphan G-protein-coupled receptor (GPR54). Proteolytic fragments of *KISS1* were named metastin and kisspeptin.

Recently, *KISS1* levels have been shown to rise in early placenta and molar pregnancies. Levels also are reduced in choriocarcinoma cell lines, favoring a predominant role for the protein in control-



CRSP3-TXNIP-KISS1 metastasis suppressor pathway. KISS1 generates proteolytic fragments, metastin and kisspentin, which bind to an orphan G-protein-coupled receptor GPR54. Ligand binding initiates intracellular signaling cascades. Upregulation of KISS1 led to decreased NFκB activation and lower MMP-9 transcription; exogenous metastin/kisspentin treatment also reduced metastasis and anchorage-independent growth.

Although KISS1 is encoded on chromosome 1, studies suggested a regulatory element of KISS1 was located on chromosome 6. Ultimately, two molecules, CRSP3 and TXNIP, were identified that appear to function upstream of KISS1.

TXNIP, also encoded on chromosome 1, binds a reduced form of thioredoxin (TRN), which regulates stress-response MAPK signaling. Increased TXNIP expression leads to decreased TRN expression and arrested cell growth.

CRSP3, located on chromosome 6, encodes a cofactor required for SP1 (specialty protein 1)-mediated activation of transcription. Transfection of *CRSP3* cDNA into melanoma cells led to increased expression of both TXNIP and KISS1 as well as suppression of metastasis, implicating it as the regulatory element.

ling invasive and migratory properties of trophoblast cells.

Recent genetic studies in humans and generation of transgenic mice have implicated GPR54 in pubertal development and pregnancy. Unfortunately, those findings have shed little light on the mechanism by which metastasis is suppressed.

Clinical studies likewise have been hampered by a lack of antibodies/antisera recognizing *KISS1*, metastin, kisspeptins, and the receptor. Nonetheless, in situ hybridization in clinical melanoma samples showed that *KISS1* expression was decreased in more advanced melanomas. In mRNA studies, tumors that had lost *KISS1* expression also showed loss of heterozygosity of loci along the long arm of chromosome 6, corroborating findings by MMCT linking to loci 6q16.3-q23.

Taking advantage of the cumulative evidence, Steven Goldberg and colleagues identified two molecules, TXNIP and CRSP3, that appear to function upstream of *KISS1*. Briefly, paired microarrays were used to compare metastatic and nonmetastatic C8161 cells. The gene with greatest differential expression was *VDUP1* (vitamin D_3 upregulated protein 1).

VDUP1 was first identified in HeLa cells after treatment with 1,25-dihydroxyvitamin-D_3. Subsequently, it was shown to interact with thioredoxin (TRN) and has become known as TRN-interacting protein (TXNIP).

TRN is a redox-signal-regulating protein that regulates stress-response MAPK signaling and also activates transcription factors to regulate stress-response apoptosis. *TXNIP*, like *KISS1*, is encoded on chromosome 1. Thus, the regulator of *KISS1* encoded on chromosome 6 was not identified immediately using the array strategy.

Concurrent positional cloning studies provided a clue. *CRSP3* encodes a cofactor required for SP1-mediated activation of transcription. Because *KISS1* and *TXNIP* promoters contain SP1 elements, it was hypothesized that *CRSP3* could be the regulatory element, because it mapped to the distal end of the minimal region identified in the 6q16.3-q23 deletion variant.

When *CRSP3* cDNA was transfected into melanoma cells, *KISS1* and *TXNIP* expression increased concomitant with suppression of metastasis. Tumorigenicity was unaffected. Thus, the first metastasis suppressor "pathway" was identified: CRSP3→TXNIP→KISS1.

TIMPs

Tissue inhibitors of metalloproteinases (TIMPs) are a family of secreted proteins that selectively inhibit MMPs. Modulation of MMP and TIMP levels controls tumor invasion and tumor-induced angiogenesis.

Yet, the situation is not simple. Paradoxically, TIMP-1, 2, and 4 exert antiapoptotic effects, whereas TIMP-3 induces apoptosis. TIMP-2, in concert with MT1-MMP, can bind to and activate proMMP-2.

TIMPs are expressed in tumor tissues and are present in the sera of cancer patients. Transfection can inhibit metastasis or tumorigenicity, depending on the cell type. The involvement of TIMPs and MMPs with the extracellular matrix provides a possible example of tumor-microenvironment interactions.

Cadherins

Cadherins are widely expressed transmembrane glycoproteins responsible for Ca^{2+}-dependent cell adhesion. E-cadherin is expressed on epithelial cells, where the extracellular domain is critical for homophilic Ca^{2+}-dependent cell–cell adhesion. The intracellular domain interacts with β-catenin to mediate actin binding.

The role of E-cadherin as a metastasis suppressor is complex. As expected, overexpression decreases tumor cell motility, invasion, and shedding from the primary tumor, thereby suppressing metastasis. However, E-cadherin can also suppress tumorigenicity in other cell types.

Interpretation of these data is complicated because downregulation of E-cadherin is often compensated by upregulation of other N-cadherins and/or cadherin-11. High levels of the latter cadherins are associated with increased invasiveness and metastasis in breast and melanoma cells. These findings highlight the complexities introduced by cell origin and cell context.

MKK4

MKK4/JNKK1/SEK1 is a mitogen-activated protein kinase that transduces signals from MEKK1 to stress-activated protein kinase/JNK1 and $p38^{MAPK}$. MKK4 transmits stress signals to nuclear transcription factors that eventually control cell proliferation, apoptosis, and differentiation.

Prostate cancer cell metastasis was suppressed after transfection of MKK4. An inverse relationship between tumor grade and MKK4 staining was found in prostate tumors and ovarian carcinoma.

BRMS1

BRMS1, which maps to chromosome 11q13, was identified from MMCT hybrids of human breast carcinoma cell lines. Following transfection into human and murine breast/mammary carcinoma cell lines and human melanoma lines, metastasis was suppressed, but tumorigenicity was not inhibited. Transfectants were not suppressed for growth in vitro or in vivo, adhesion to extracellular matrix components, expression of MMPs or heparanase, or invasion.

Interestingly, one of the metastasis efficiency loci mapped by Kent Hunter and colleagues is at a region syntenic to *Brms1* in mice. Other studies utilizing comparative sequence analysis, however, suggest that *Brms1* may not be the only gene in the region.

BRMS1 transfectants restored intercellular communications via gap junctions, concomitant with increased expression of connexin Cx43 and decreased expression of Cx32. Connexins are the protein subunits of gap junctions, and the expression pattern in *BRMS1* transfectants was more reminiscent of normal breast tissue.

Recent data show that BRMS1 is part of a complex containing histone deacetylases (HDACs). Based on the roles of HDACs in regulating chromatin structure and corresponding changes in gene expression, it would appear that BRMS1

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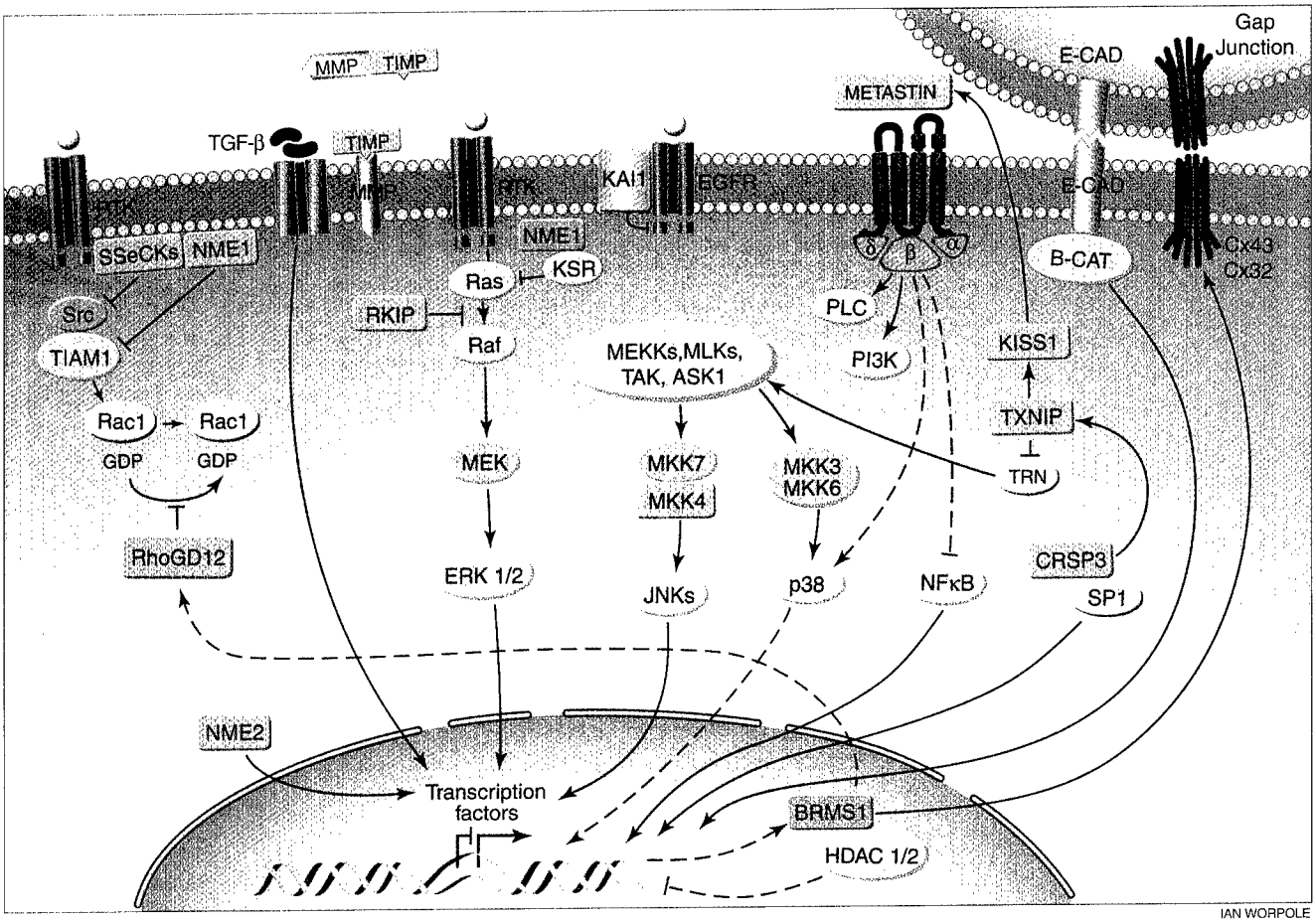

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Thirteen metastasis suppressor genes (*purple*) have been cloned to date for which functional evidence has been obtained, and others are suspected. The *solid lines* depict pathways for which biochemical evidence exists, and *dotted lines* represent inferred pathways. Within these same suppressor pathways, several metastasis promoters, such as TGF-β, may also function.

controls metastasis by regulating gene expression.

SSeCKS

SSeCKS (pronounced *essex*), for Src-suppressed C kinase substrate expression, is downregulated in *src*- and *ras*-transformed rodent fibroblasts. It is the rodent ortholog of human Gravin/KAP12, a cytoplasmic scaffold protein for protein kinases A and C, concentrating at the cell edge and podosomes (ie., cytoplasmic extensions).

SSeCKS is detectable in untransformed prostate epithelial cells, but expression is reduced in metastatic prostate carcinoma cell lines. Re-expression reduces lung metastasis, induces formation of filopodia-like projections, and decreases anchorage-independent growth in vitro.

RhoGDI2

RhoGDI (Rho GDP dissociation inhibitors) are guanine nucleotide-binding proteins, which cycle between the active GTP-bound state and inactive GDP-bound state. It stabilizes the GDP-bound form and sequesters them in an inactive, nonmembrane-localized, cytoplasmic compartment.

mRNA expression of RhoGDI2 was associated with decreased metastatic potential in bladder carcinomas. Transfection and enforced expression suppressed metastasis of T24 human bladder carcinoma variants. Gene expression profiling of 105 bladder carcinomas further corroborated the expression pattern—i.e., RhoGDI2 expression correlated inversely with the invasive phenotype of tumors.

Drg-1

Drg-1 (also termed RTP, cap43, and rit42) is a differentiation-associated gene in colon carcinomas. Although it has tumor suppression capacity in human bladder carcinoma, metastasis but not tumorigenicity was suppressed in prostate carcinoma cells.

METASTASIS SUPPRESSOR GENES

Gene	Function
NM23	Histidine kinase; phosphorylates KSR and reduces ERK1/2 activation in response to signaling
TIMPs	?
E-Cadherins	Cell-cell adhesion
KAI1 (CD82)	A tetraspanin; possibly involved in integrin interaction/adhesion and EGFR desensitization
KISS1	G-protein-coupled receptor ligand (metastin or kisspeptin)
MKK4	MAPKK; phosphorylates and activates p38 and JNK kinase
BRMS1	Gap-junctional communication; chromatin structure
CRSP3	Transcriptional coactivator
VDUP1 (*TXNIP*)	Thioredoxin (TRN) inhibitor
RHOGDI2	Regulates Rho and Rac function
SSeCKs	?
Drg-1	?

ADAPTED FROM BOGENRIEDER T, HERLYN M: *ONCOGENE* 22:6524-6536, SEP 29 2003.

MMCT has been the most fruitful technique for identifying metastasis suppressor genes, but use of other approaches is growing. Discoveries based on techniques such as subtractive hybridization, differential display, and microarray are becoming more frequent.

Metastasis Suppressor Genes Have Been Cloned

To date, at least 13 genes have been cloned for which functional evidence demonstrates metastasis suppressor activity. The gene products exist in every cellular compartment, highlighting multiple points of control. Still, however, the mechanism of action remain unknown. Recent experiments are starting to provide initial clues.

Nm23

In 1986, Patricia Steeg and coworkers screened cDNA libraries of matched metastatic/nonmetastatic K1735 murine melanoma cell lines by differential hybridization and identified "nonmetastatic clone 23" (*Nm23*) gene as the first metastasis suppressor gene. Enforced expression of this gene in cell lines of diverse cellular origin suppressed metastasis without altering tumor growth.

The human ortholog, *NM23-H1* (also termed *NME1*), is a nucleoside diphosphate kinase, although activity of this kinase is not responsible for antimetastatic activity. Eight other family members have since been identified. Of this family, *NM23-H1* and *NM23-H2* appear to have metastasis suppressor activity.

Building on prior data showing that Nm23 histidine kinase activity is responsible for metastasis suppression, it was found that Nm23 forms a complex with kinase suppressor of Ras (KSR), a scaffold protein for the mitogen-activated protein kinase (MAPK) cascade.

Most studies show decreased expression of *NM23* in clinical tumor tissues (as would be expected for an unactivated metastasis suppressor). Higher expression has been noted consistently in neuroblastomas, suggesting that *NM23* functions differently in neuroblasts than in other cells.

KAI1 (CD82)

KAI1 was identified in rat prostate cancer cell lines that showed metastasis suppression following MMCT of human chromosome 11. *KAI1* maps to chromosome 11p11.2 and is an evolutionarily conserved glycoprotein of the tetraspanin trans-

Consistent with the transfection studies, *Drg-1* expression was inversely correlated with Gleason score in human prostate cancer specimens. The mechanism of action is unknown, although *Drg-1* may function downstream of MKK4, because it is induced similarly to the stress-activated protein kinases (JNK/SAPK).

Newer Suppressors

The number of metastasis suppressors and presumptive metastasis suppressors continues to grow. Several molecules have shown evidence that they can function as metastasis suppressors, but their characterization is still incomplete. For the most part, they have not been tested in vivo for metastasis suppression, and so, they cannot be definitively assigned as metastasis suppressors.

The list of presumed suppressors includes semaphorins, CRMP-1, gelsolin, maspin, HP1$^{HS\alpha}$, CD44, and SHP-2.

Metastasis genetics is a relatively young, emerging field of study. As metastasis-associated genes are identified, patterns are beginning to emerge that provide insights into the underlying mechanisms controlling cancer spread. The picture is still sketchy and will evolve; but some common elements are apparent.

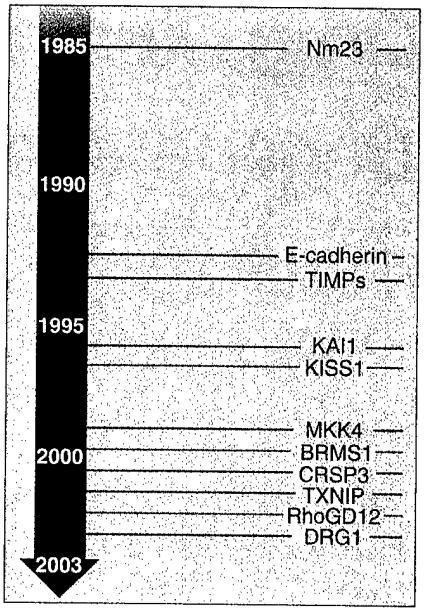
First, metastasis suppressors are found in every cellular compartment and appear to be in positions that would amplify "signals" (i.e., there are several branches downstream in each signaling arbor). This situation is to be expected for complex, multigenic phenotypes such as metastasis.

Second, many metastasis suppressor genes discovered in one tumor type also suppress metastasis in cells of other origins, suggesting that common pathways for metastasis control are shared by tumors of different histologic origin.

Third, despite use of a strict definition of metastasis suppression (i.e., suppression of metastasis without inhibition of tumorigenicity), there are increasing examples of effects of metastasis suppressors on tumorigenicity depending on cell type. Given the complexity of cancer biology, this is not expected. These data point to an extremely important parameter affecting tumor cells—cellular context.

Finally, many of the metastasis suppressors seem to block growth of cells at secondary sites. The observation that tumor cells grow at orthotopic sites but not at metastatic sites implies that metastasis suppressors are mediators of cellular context. The finding also suggests that metastasis genes may regulate metastasis to some sites, but not others.

Taken together, an accumulating body of data shows the existence of pro- and antimetastatic genes. The number and nature of these genes continues to grow, while their interrelationships and mechanisms of action become elucidated. As the field moves forward, there is optimism that these genes can become targets for therapeutic intervention.



Since the identification of *Nm23* in the mid-1980s, the list of metastasis suppressor genes has grown rapidly.

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